PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
C12N 15/82, 9/90, A01H 5/08, 5/10, A61K 35/78, A23L 1/24, 1/39, A61K 7/48

A1

(11) International Publication Number: **WO 00/04175**

(43) International Publication Date: 27 January 2000 (27.01.00)

(21) International Application Number: PCT/EP99/05027

(22) International Filing Date: 13 July 1999 (13.07.99)

(30) Priority Data:
98305570.8 14 July 1998 (14.07.98) EP

(71) Applicant *(for AE AU BB CA CY GB GD GH GM IE IL KE LC LK LS MN MW NZ SD SG SL SZ TT UG ZA ZW only)*: UNILEVER PLC [GB/GB]; Unilever House, Blackfriars, London EC4P 4BQ (GB).

(71) Applicant *(for all designated States except AE AU BB CA CY GB GD GH GM IE IL IN KE LC LK LS MN MW NZ SD SG SL SZ TT UG ZA ZW)*: UNILEVER NV [NL/NL]; Weena 455, NL-3013 AL Rotterdam (NL).

(71) Applicant *(for IN only)*: HINDUSTAN LEVER LIMITED [IN/IN]; Hindustan Lever House, 165/166 Backbay Reclamation, Mumbai 400 020, Maharashtra (IN).

(72) Inventors: BOVY, Arnaud, Guillaume; Waterweg 48B, NL-3731 HM De Bilt (NL). HUGHES, Stephen, Glyn; 70 Ashdon Road, Saffron Walden, Essex CB10 2AL (GB). MUIR, Shelagh, Rachael; Unilever Research Colworth, Colworth House, Sharnbrook, Bedford MK44 1LQ (GB). VAN TUNEN, Adrianus, J.; Wim Sonneveldstraat 31, NL-6708 NA Wageningen (NL). VERHOEYEN, Martine, Elisa; Unilever Research Colworth, Colworth House, Sharnbrook, Bedford MK44 1LQ (GB). DE VOS, Cornelis, Henricus; Caro van Eyckstraat 15, NL-6708 NH Wageningen (NL).

(74) Agent: EVANS, Jacqueline, Gail, Victoria; Unilever PLC, Patent Dept., Colworth House, Sharnbrook, Bedford MK44 1LQ (GB).

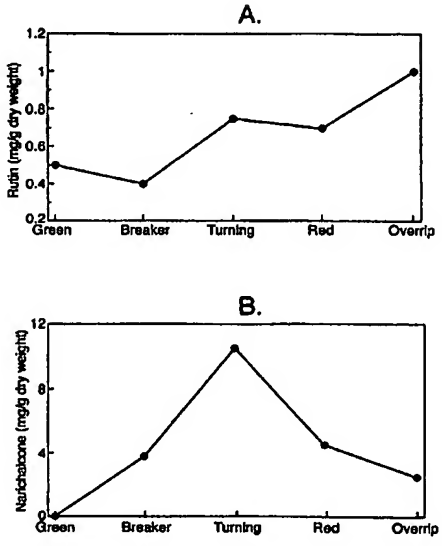
(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHODS AND COMPOSITION FOR MODULATING FLAVONOID CONTENT

(57) Abstract

A method for manipulating the production of flavonoids in tomatoes by manipulating gene activity in the flavonoid biosynthetic pathway by expressing genes encoding chalcone isomerase, compositions for use in such a method and tomato plants having altered flavonoid levels are disclosed.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Paso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

METHODS AND COMPOSITION FOR MODULATING FLAVONOID CONTENT

FIELD OF THE INVENTION

The present invention relates generally to methods for manipulating the production of flavonoids in plants by manipulating endogeneous and incorporated gene activity in the flavonoid biosynthetic pathway and compositions for use in such methods. In particular, it relates to methods for increasing flavonoid levels by altering the level of chalcone isomerase activity. Chalcone isomerase is an enzyme involved in the biosynthetic pathway of flavonoids.

BACKGROUND OF THE INVENTION

Flavonoids form a large group of polyphenolic compounds, based on a common diphenylpropane skeleton, which occur naturally in plants. Included within this class of compounds are flavonols, flavones, flavanones, catechins, anthocyanins, isoflavonoids, dihydroflavonols and stilbenes. The flavonoids are mostly present as glycosides.

In tomato fruits, the main flavonoid found is naringenin chalcone (Hunt et al, Phytochemistry, 19, (1980), 1415-1419). It is known to accumulate almost exclusively in the peel and is simultaneously formed with colouring of the fruit. In addition to naringenin chalcone, glycosides of quercetin and, to a lesser extent, kaempferol are also found in tomato peel.

Reports in the literature suggest that there is increasing evidence that flavonoids are potentially health-protecting components in the human diet. Epidemiological studies suggest a direct relationship between cardioprotection and increased consumption of flavonoids, in particular flavonols of the quercetin and kaempferol type, from dietary sources such as onion,

apples and tea (see, for example, Hertog et al, Lancet, 342 (1993), 1007-1011).

Flavonoids have been reported to exhibit a wide range of biological activities *in vitro* including anti-inflammatory, anti-allergic and vasodilatory activity (Cook et al, Nutritional Biochemistry, 7, (1996), 66-76). Such activity has been attributed in part to their ability to act as antioxidants, capable of scavenging free radicals and preventing free radical production. Within this group of compounds, those having the most potent antioxidant activity are the flavonols (Rice-Evans et al, Free Radical Research, 22, (1995), 375-383). In addition, flavonoids can also inhibit the activity of key processes such as lipid peroxidation, platelet aggregation and capillary permeability (see Rice-Evans et al, Trends in Plant Science, 2, (1997), 152-159).

Based on studies of this type, there is presently considerable interest in the development of food products from plants rich in such protective flavonoids.

It would be desirable to produce plants which intrinsically possess elevated levels of health protecting compounds such as flavonoids in order to develop food products with enhanced protective properties. Traditionally, the approach to improving plant varieties has been based on conventional cross-breeding techniques, but these are slow as they require time for breeding and growing successive plant generations. More recently, recombinant DNA technology has been applied to the general problem of modifying plant genomes to produce plants with desired phenotypic traits. Whilst reference has been made in the literature to the use of genetic manipulation techniques in modifying the flavonoid biosynthetic pathway, as discussed beneath, it is notable that these attempts have been directed in general towards modifying pigmentary anthocyanin production.

The flavonoid biosynthetic pathway is well established and has been widely studied in a number of different plant species (see, for example, Koes et al, BioEssays, 16, (1994), 123-132). Briefly, three molecules of malonyl-CoA are condensed with one molecule of Coumaroyl-CoA, catalysed by the enzyme chalcone synthase, to give naringenin chalcone which rapidly isomerises, catalysed by chalcone isomerase, to naringenin. Subsequent hydroxylation of naringenin catalysed by flavanone 3-hydroxylase leads to dihydrokaempferol. Dihydrokaempferol itself can be hydroxylated to produce either dihydroquercetin or dihydromyricetin. All three dihydroflavonols subsequently can be converted to anthocyanins (by the action of dihydroflavonol reductase and flavonoid glucosyltransferase) or alternatively converted to flavonols such as kaempferol, quercetin and myricetin by the action of flavonol synthase.

A schematic overview of the flavonoid biosynthetic pathway is presented in appendix 1, figure 1.1.

The manipulation of flavonoid levels in plants by altering the expression of a single flavonoid biosynthetic gene is disclosed by Napoli (1990, Plant Cell, 2:279-289). Napoli discloses the introduction of a chimeric chalcone synthase (CHS) gene into Petunia. Said introduction is described to result in a block in the anthocyanin biosynthesis. The resulting transformed petunia plants therefore contained lower levels of flavonoids than untransformed plants, presumably due to co-suppression of the endogeneous CHS activity.

Que (1997, Plant Cell, 9: 1357-1368) discloses a comparison of the effect of strong and weak promoters that drive sense chalcone synthase transgenes in large populations of independently transformed plants. It is shown that a strong transgene promoter is required for high frequency cosuppression of CHS genes and for the production of a full range of phenotypes.

Howles (1996, Plant Physiol. 112: 1617-1624) discloses the stable genetic transfer of the flavonoid biosynthetic gene phenylalanine ammonia-lyase (PAL) from french bean into tobacco. A proportion of the obtained transgenic tobacco plants is shown to display overexpression of PAL activity. According to Howles PAL overexpressing plants do not contain altered levels of flavonoids.

It has been disclosed by Tanaka et al (1995, Plant and Cell Physiology 36: 6, 1023-1031) that heterologous transformation of dihydroflavonol reductase (DFR) can be used for the production of plants with altered levels of anthocyanins.

There is no disclosure in the literature of the manipulation of flavonoids in plants by means of overexpression of chalcone isomerase.

Accordingly, there remains a continuing need for the development of methods for enhancing the levels of flavonoids, in particular flavonols, in plants.

SUMMARY OF THE INVENTION

Therefore, in a first aspect, the invention provides a method for producing a plant capable of exhibiting altered levels of flavonoids comprising incorporating into said plant one or more gene sequences encoding a protein with chalcone isomerase activity, or incorporating a nucleotide sequence encoding a protein functionally equivalent thereto.

The invention also provides a plant having one or more transgenes each encoding a protein with chalcone isomerase activity, or a protein functionally equivalent thereto, incorporated into its genome such that its ability to produce flavonoids is altered.

According to a highly preferred embodiment, the invention further provides a tomato plant having one or more transgenes each encoding a protein with chalcone isomerase activity, or a protein

functionally equivalent thereto, incorporated into its genome such that its ability to produce flavonoids is altered.

Also provided is a transformed plant having enhanced flavonoid levels, not being chalcones, particularly enhanced flavonol levels compared to similar untransformed plants. Preferably the level of said flavonoids, not being chalcones, in transformed plants is at least 4 times higher than in similar untransformed plants, more preferred 5-100, most preferred 10-40 times higher than in similar untransformed plants.

Further provided is a fruit-bearing plant, particularly a tomato plant, having flavonoids, particularly flavonols, in the peel of the fruit.

Seeds, fruits and progeny of such plants and hybrids are also included within the invention.

The invention further provides DNA constructs coding for a protein with chalcone isomerase activity, or a functionally equivalent sequence of said DNA construct, operably linked to a promoter. When transformed into a plant cell, these constructs are useful for overexpressing genes encoding proteins with chalcone isomerase activity, thereby altering the ability of the plant to produce flavonoids. The invention also provides for plants comprising these constructs together with seeds, fruits and progeny thereof.

Food products such as sauces, dressings, ketchups and soups, comprising at least part of a plant prepared according to the invention are also provided.

Also provided are skin and hair protective products comprising at least part of a plant according to the invention.

Also provided are pharmaceuticals comprising at least part of a plant according to the invention.

Definition of terms

As used herein, "plant" means a whole plant or part thereof, or a plant cell or group of plant cells. It will be appreciated that also extracts are comprised in the invention.

A "flavonoid" or a "flavonol" may suitably be an aglycon or a conjugate thereof, such as a glycoside, or a methyl, acyl, sulfate derivative.

A "protein with chalcone isomerase activity" is a protein being capable of enzymatically catalysing the conversion of a chalcone into a flavanone, for example narichalcone into naringenin.

A "gene" is a DNA sequence encoding a protein, including modified or synthetic DNA sequences or naturally occurring sequences encoding a protein, and excluding the 5' sequence which drives the initiation of transcription.

A "DNA sequence functionally equivalent thereto" is any sequence which encodes a protein which has similar functional properties.

According to another embodiment, a functionally equivalent DNA sequence shows at least 50 % similarity to the respective DNA sequence. More preferably a functionally equivalent DNA sequence shows at least 60%, more preferred at least 75%, even more preferred at least 80%, even more preferred at least 90%, most preferred 95-100% similarity, to the respective DNA sequence.

According to the most preferred embodiment a functionally equivalent DNA sequence shows not more than 5 base pairs difference to the respective DNA sequence, more preferred less than 3, e.g. only 1 or 2 base pairs different.

According to another embodiment a functionally equivalent sequence is preferably capable of hybridising under low stringent conditions to the respective sequence.

"Breaker" is the ripening stage corresponding to the appearance of the first flush of colour on the green fruit.

"Operably linked to one or more promoters" means the gene, or DNA sequence, is positioned or connected to the promoter in such a way to ensure its functioning. The promoter is any sequence sufficient to allow the DNA to be transcribed. After the gene and promoter sequences are joined, upon activation of the promoter, the gene will be expressed.

A "construct" is a polynucleotide comprising nucleic acid sequences not normally associated in nature.

An "altered" level of flavonoids is used throughout this specification to express that the level of specific flavonoids in the transformed plants differs from the level of flavonoids present in untransformed plants. Preferably the difference is between 0.1 and 100 fold. It will be appreciated that the specific flavonoids as meant here are flavonoids other than chalcones as said specific flavonoids are formed at the expense of chalcones.

Therefore in the specification where these flavonoids are meant reference will be made to "specific flavonoids".

An "increased" level of flavonoids is used to indicate that the level of is preferably at least 4 times higher than in similar untransformed plants, more preferred 5-100, most preferred 10-40 times higher than in similar untransformed plants.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention may be more fully understood by reference to the following description, when read together with the accompanying drawings in which:

Figure 1 shows the levels of the two dominant flavonoids, rutin (A.) and narichalcone (B.) in FM6203 tomato peel during ripening. Results represent the means of three independent samples.

Figure 2 shows the northern analysis of tomato fruit harvested at different developmental stages, denoted as: green (G), breaker (B), turning (T) and red (R), and separated into peel and flesh. Leaves (L) were harvested from young tomato plants. RNA was isolated from the samples, separated on formaldehyde-agarose gels, blotted and hybridised with petunia *chs-a*, *chi* and *fls* probes.

Figure 3 shows the restriction maps of pFLAP10 and pFLAP50.

Figure 4 shows the restriction maps of pBBC3, pBBC50 and pSJ89.

Figure 5 shows the Southern blot of chromosomal DNA from tomato. Chromosomal DNA was isolated from young leaves of transgenic and non-transgenic tomato plants. 10 µg DNA was digested with EcoRI, separated on an agarose gel and blotted onto a nylon filter. The DNA was hybridised with a ^{32}P-labelled *npt*II specific probe and autoradiographed.

Figure 6 shows typical HPLC chromatograms, recorded at 370 nm, of hydrolysed extracts of (A.) peel and (B.) flesh tissue of plants transformed with the control plasmid pSJ89. Peaks corresponding to the quercetin and kaempferol aglycons are indicated.

Figure 7 shows a typical HPLC chromatogram, recorded at 360 nm, of a non-hydrolysed extract of peel tissue of a tomato plant transformed with the control plasmid pSJ89. Peaks corresponding to rutin, quercetin trisaccharide and narichalcone are indicated.

Figure 8 shows levels of quercetin in hydrolysed extracts of flesh of tomatoes transformed with either the control pSJ89 (G series of transformed plants) or the pBBC50 (C series of transformed plants) gene constructs.

Figure 9 shows a typical HPLC chromatogram, recorded at 360 nm, of a non-hydrolysed extract of peel tissue of a tomato plant transformed with pBBC50 (plant number C87). The major peaks correspond to rutin (R), isoquercitrin (IQ), kaempferol rutinoside/quercetin glycoside (KR/QG)(co-eluting compounds) and a putative kaempferol glycoside (KG) are marked.

Figure 10 shows the proposed biosynthetic pathway for the production of flavonoids.

Figure 11 shows the graph of the data represented in table 2.

Figure 12 shows restriction maps of plasmids pUCAP and pUCM2.

Figure 13 shows the restriction map of plasmid pFLAP10.

Figure 14. shows the multiple cloning site as altered in pUCM2 from AscI to PacI in the 5' to 3' orientation.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the unexpected finding that chalcone isomerase may be a rate limiting step in the production of flavonoids in tomato fruit.

We have surprisingly found that upon incorporation of a gene sequence encoding for a protein with chalcone isomerase activity in plants, the subsequent overexpression of this protein leads to very high (sometimes even 50-100 fold) increase in the amount of flavonoids in the fruit of said plant.

Applicants have found that in ripening tomato fruit two dominant flavonoids can be detected: flavonol rutin and narichalcone, which both accumulate in the peel of tomato fruit. At no developmental stage were significant amounts of flavonoids detected in the flesh of fruit. Without wishing to be bound by any theory applicants believe that the accumulation of narichalcone in the peel of fruit before declining through the red and over ripe stages, is indicative that chalcone isomerase represents a rate limiting step in the formation of flavonoids.

A method for elucidation of the rate limiting step in flavonoid biosynthesis is further illustrated in the examples.

Advantageously, by means of the invention, levels of specific flavonoids, more particularly flavonols, in plants, particularly tomatoes, may be altered. Preferably in the method according to the invention the levels of flavonoids, more particularly flavonols, in plants, particularly tomatoes, are increased. Moreover, it has been found that the level of flavonoids, in particular the level of specific flavonols, may be increased specifically in the peel of tomato fruit, thereby producing tomatoes with enhanced nutritional, preservative and flavour characteristics.

Most preferred in the method according to the invention the transformed plant exhibits increased levels of kaempferol and/or quercetin, or their glycosides or derivatives thereof.

It will be appreciated that the invention furthermore relates to a method for producing a plant capable of exhibiting altered levels of flavonoids, comprising incorporating into said plant a gene sequence encoding for chalcone isomerase, thereby increasing the level of flavonoids by overexpression of said chalcone isomerase. Therefore it will be understood that the invention encompasses said gene sequence encoding for chalcone isomerase and any sequence functionally equivalent thereto. This group of sequences

is in the course of this application also referred to as "a gene comprising a nucleotide sequence encoding an enzyme with chalcone isomerase activity".

Therefore according to a further embodiment the invention relates to a method for producing a plant capable of exhibiting altered levels of flavonoids comprising incorporating into said plant a gene comprising a nucleotide sequence encoding an enzyme with chalcone isomerase activity.

According to a preferred embodiment said gene comprises a nucleotide selected from:

(i) a nucleotide sequence, encoding an amino acid sequence having at least 40 % similarity, to seq ID No1;

(ii) a nucleotide sequence capable of hybridising under low stringent conditions to a sequence selected from the group of sequences set forth under (i) above;

(iii) a nucleotide sequence encoding a protein that is functionally equivalent to the protein encoded by seq ID no 1.

Seq, ID 1 is an amino acid sequence obtainable from PIR database, accession number SO4725, as published by van Tunen et al, EMBO J. 7, 1257-63 1988.

More preferred said gene comprises the nucleotide sequence encoding an amino acid sequence having at least 60% similarity preferably at least 90%, more preferred at least 95% or even 98%, similarity to the sequence as set forth in seq ID No1 (amino acid sequence of chalcone isomerase).

According to a highly desired embodiment, the gene which is incorporated into the plant in the method according to the invention encodes the amino acid sequence of chalcone isomerase from petunia as set forth in seq ID No 1.

According to a preferred embodiment said nucleotide sequence comprises a sequence which has at least 50% similarity, more preferred at least 60%, even more preferred at least 75%, even more preferred at least 80%, still more preferred at least90 %, most preferred at least 95% or even 98-100% similarity to Sequence ID no 2, and whereby said sequence encodes a protein having chalcone isomerase activity.

Although the percentage similarity referred to above assumes an overall comparison between the sequence set forth in at least one of the sequences of Seq ID 1, Seq ID 2, it is clear that there may be specific regions within molecules being compared, having less than 60% similarity.

It will be appreciated that the invention extends to any plant which is amenable to transformation.

Therefore, according to another embodiment, the invention repates to a plant having one or more transgenes, each encoding a protein with chalcone isomerase activity or a protein functionally equivalent thereto, incorporated into its genome such that its ability to produce flavonoids is altered.

Preferably the plants according to the invention are suitable for human consumption. Suitable plants are for example vegetables, fruits, nuts, herbs, spices, infusion materials. Suitable vegetables are for example from the Pisum family such as peas, family of Brassicae, such as green cabbage, Brussel sprouts, cauliflower, the family of Phaseolus such as barlotti beans, green beans, kidney beans, the family of Spinacea such as spinach, the family of Solanaceae such as potato and tomato, the family of Daucus, such as carrots, family of Capsicum such as green and red pepper, and berries for example from the family of Ribesiaceae, Pomaceae, Rosaceae, for example strawberries, black berries, raspberries, black current and edible grasses from the family of Gramineae such as maize, and citrus fruit for example from the

family of Rutaceae such as lemon, orange, tangerine. Also preferred are plants which can form the basis of an infusion such as black tea leaves, green tea leaves, jasmin tea leaves. Also preferred is the tobacco plant.

A particularly preferred plant for use in the method according to the invention is the tomato plant.

It will furthermore be appreciated that the sequence encoding a protein with chalcone isomerase activity may be a genomic or cDNA clone, or a sequence which in proper reading frame encodes an amino acid sequence which is functionally equivalent to the amino acid sequence of the protein encoded by the genomic or cDNA clone. By "functionally equivalent" is meant any DNA sequence which is capable of similar biological activity. A functional derivative can be characterised by an insertion, deletion or a substitution of one or more bases of the DNA sequence, prepared by known mutagenic techniques such as site-directed mutagenesis. The functionality can be evaluated by routine screening assays, for example, by assaying the flavonoid content of the resulting transgenic plant. An in vitro assay to determine chalcone isomerase activity has been described by van Weely (1983, *Planta* 159: 226-230).

Gene sequences encoding a gene sequence for proteins with chalcone isomerase activity for use according to the present invention may suitably be obtained from plants, in particular higher plants as these generally possess a flavonoid biosynthetic pathway. Suitable gene sequences can for example be obtained from petunia, maize, arabidopsis, alfalfa, pea, bean, grape, apple.

The gene sequences of interest are preferably operably linked (that is, positioned to ensure the functioning of) to one or more suitable promoters which allow the DNA to be transcribed. Said promoters are preferably promoters useful to obtain over-expression of the protein with chalcone isomerase activity in said host plant. Suitable promoters, which may be homologous or

heterologous to the gene (that is, not naturally operably linked to the expressed gene encoding a chalcone isomerase protein or a functional equivalent thereof) useful for expression in plants are well known in art, as described, for example, in Weising et al, (1988), Ann. Rev. Genetics, 22, 421-477). Promoters for use according to the invention may be inducible, constitutive or tissue-specific or have various combinations of such characteristics. Useful promoters include, but are not limited to, constitutive promoters such as carnation etched ring virus (CERV), cauliflower mosaic virus (CaMV) 35S promoter, or more particularly the enhanced cauliflower mosaic virus promoter, comprising two CaMV 35S promoters in tandem (referred to as "Double 35S"), or the GBSS (granular bound starch synthase) promoter.

According to a preferred embodiment fruit specific promoters are used. Suitable fruit-specific promoters include the tomato E8 promoter (Deikman et al, (1988), EMBO J, 7, 3315-3320), 2A11 (Van Haaren et al, Plant Mol Biol, 21, 625-640), E4 (Cordes et al, (1989), Plant Cell, 1, 1025-1034) and PG (Bird et al, (1988), Plant Mol. Biol., 11, 651-662,) Nicholass et al, (1995), Plant Molecular Biology, 28, 423-435, pTOM96 (ref), fpb11(WO-A-91/05054).

In another preferred embodiment, the promoter is a constitutive enhanced 35S CaMV promoter.

It will be appreciated that accumulation of flavonoids may be inhibited by the rate of production of the amino acid phenylalanine, the primary substrate in the synthesis of phenylpropanoids and subsequent flavonoids. In order to increase phenylalanine biosynthesis, genes encoding enzymes of the phenylalanine pathway that are insensitive to feed-back regulation may be introduced as an optional additional step.

Preferably the desired gene sequences, operably linked to respective suitable promoters, are fused to appropriate expression

sequences to provide an expression cassette functional in a plant cell which can be introduced into a plant cell by any conventional plant transformation method.

Therefore the invention also relates to a DNA construct comprising sequences encoding for a protein with chalcone isomerase activity, or a functionally equivalent sequence thereof, operably linked to a promoter; and relates to plants, preferably tomato plants comprising said DNA construct.

Accordingly, the invention provides in a further aspect an expression cassette comprising as operably linked components in the 5'-3' direction of transcription at least one unit, comprising a promoter functional in a plant cell, a gene sequence encoding a protein with chalcone isomerase activity and a transcriptional termination regulatory region functional in a plant cell.

The promoter and termination regulatory regions will be functional in the host plant cell and may be heterologous (that is, not naturally occurring) or homologous (derived from the plant host species) to the plant cell and the gene. Suitable promoters which may be used are described above.

The termination regulatory region may be derived from the 3' region of the gene from which the promoter was obtained or from another gene. Suitable termination regions which may be used are well known in the art and include *Agrobacterium tumefaciens nopaline synthase terminator (Tnos), Agrobacterium tumefaciens mannopine synthase terminator (Tmas)* and the *CaMV 35S terminator (T35S)*. Particularly preferred termination regions for use according to the invention include the tobacco ribulose bisphosphate carboxylase small subunit termination region (TrbcS) or the *Tnos* termination region.

Such gene constructs may suitably be screened for activity by transformation into a host plant via *Agrobacterium* and screening for flavonoid levels.

Conveniently, the expression cassette according to the invention may be prepared by cloning the individual promoter/gene/terminator unit into a suitable cloning vector. Suitable cloning vectors are well known in the art, including such vectors as pUC (Norrander et al, (1983, Gene 26, 101-106), pEMBL (Dente et al (1983), Nucleic Acids Research, 11, 1645-1699), pBLUESCRIPT (available from Stratagene), pGEM (available from Promega) and pBR322 (Bolivar et al, (1977), Gene, 2, 95-113). Particularly useful cloning vectors are those based on the pUC series. The cloning vector allows the DNA to be amplified or manipulated, for example, by adding sequences. The cloning sites are preferably in the form of a polylinker, that is a sequence containing multiple adjacent restriction sites, so as to allow flexibility in cloning.

In a particularly preferred embodiment, the individual promoter/gene/terminator units are cloned into adjacent pairs of restriction sites in a suitable cloning vector.

Suitably, the nucleotide sequences for the genes may be extracted from any nucleotide database and searched for restriction enzymes that do not cut. These restriction sites may be added to the genes by conventional methods such as incorporating these sites in PCR primers or by sub-cloning.

Preferably the DNA construct according to the invention is comprised within a vector, most suitably an expression vector adapted for expression in an appropriate host (plant) cell. It will be appreciated that any vector which is capable of producing a plant comprising the introduced DNA sequence will be sufficient.

Suitable vectors are well known to those skilled in the art and are described in general technical references such as Pouwels et al, Cloning Vectors. A laboratory manual, Elsevier, Amsterdam

(1986). Particularly suitable vectors include the Ti plasmid vectors.

Transformation techniques for introducing the DNA constructs according to the invention into host cells are well known in the art and include such methods as micro-injection, using polyethylene glycol, electroporation, or high velocity ballistic penetration. A preferred method for use according to the present invention relies on agrobacterium - mediated transformation.

After transformation of the plant cells or plant, those plant cells or plants into which the desired DNA has been incorporated may be selected by such methods as antibiotic resistance, herbicide resistance, tolerance to amino-acid analogues or using phenotypic markers.

Various assays may be used to determine whether the plant cell shows an increase in gene expression, for example, Northern blotting or quantitative reverse transcriptase PCR (RT-PCR). Whole transgenic plants may be regenerated from the transformed cell by conventional methods. Such transgenic plants having improved flavonoid levels may be propagated and crossed to produce homozygous lines. Such plants produce seeds containing the genes for the introduced trait and can be grown to produce plants that will produce the selected phenotype.

In accordance with a particular embodiment of the invention, the cloning vectors plasmid pUCM2 and pUCM3 were prepared by modifying the cloning vector pUCAP (Van Engelen et al, (1995), Transgenic Research, 4, 288-290).

The invention furthermore relates to a plant having one or more transgenes each encoding a protein with chalcone isomerase activity, or a sequence functionally equivalent thereto, incorporated into its genome such that its ability to produce flavonoids is altered.

The invention also encompasses a tomato plant prepared according to the method of the invention.

The following examples are provided by way of illustration only.

DNA manipulations were performed using standard procedures well known in the art, as described, for example, in Sambrook et al, Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbour Laboratory Press, 1989 (hereinafter "Sambrook").

The following literature references are mentioned in the Examples:

Becker, D. et al. (1992) Plant Mol. Biol. 20: 1195-1197
Bovy, A.G. et al. (1995) Acta Hortic. 405: 179-189.
Fulton, T.M. et al. (1995) Plant Mol. Biol. Rep. 13: 225-227
Hanahan, D. (1983) J. Mol. Biol. 166: 557-580.
Hertog, M.G.L. et al. (1992) J. Agric. Food Chem. 40: 1591-1598.
Hoekema, A. et al. (1985) Plant Mol. Biol. 5: 85-89
Jefferson, R. et al. (1987) Embo J. 6: 3901-3907
Loyd,A. et al (1992), Science 258, 1773-1775
Murashige, T. and Skoog, F. (1962) Physiol. Plant. 15: 73-97
Sambrook, J. et al. (1989) Molecular Cloning. A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Saul, M.W. et al. (1988) Plant Mol. Biol. Man. A1: 1-16 (Eds. Gelvin S.B. and Schilperoort, R.A.) Kluwer Academic Pubs., London
Symmans et al (1990) Biotechnology 8, 217-221
Vancanneyt, G. et al (1990). Mol. Gen. Gen. 220, 245-250.
Van Engelen, F. et al. (1995) Transgenic R. 4: 288-290
VanTunen, A.J. et al. (1988) EMBO J. 7: 1257-1263

EXAMPLES

Example 1: Plant Material.

Plants of tomato line FM6203 and transformants are grown in soil in a glasshouse with a 16 hour photoperiod and a 23/18 °C day/night temperature.

Example 2: Bacterial strains.

The *Escherichia coli* strain used is:

DH5α *sup*E44, (*lac* ZYA-*Arg*F)U169, 80*lac*ZM15, *hsd*R17 (rk-, mk+), *rec*A1, *end*A1, *gyr*A96, *thi*-1, *rel*A1, *deo*R (Hanahan, 1983).

The *Agrobacterium* strain used is LBA4404 (Hoekema, 1985).

Transformation of *E. Coli* DH5α is performed using the method of Hanahan, (1983).

Transformation of *Agrobacterium* LBA4404 is performed using a freeze/thaw method according to Saul *et al*, (1988).

Example 3: Elucidation of the rate-limiting step in flavonol production in tomato fruit.

The rate-limiting step in flavonol production in tomato fruit is determined using two complimentary approaches; high performance liquid chromatography (HPLC) analysis of flavonoids in ripening tomato fruit and northern analysis using probes for the flavonoid biosynthetic genes *chalcone synthase* (*chs*), *chalcone isomerase* (*chi*) and *flavonol synthase* (*fls*).

3.1 Analysis of flavonoids in ripening tomato fruit by HPLC

3.1.1 Harvest of tomato fruit

Tomato fruit are harvested at five stages of ripening (green, breaker (the ripening stage corresponding to the appearance of the first flush of color on the green fruit), turning, red and over-ripe; corresponding to approximately 21, 28, 31, 46 and 55 days post anthesis respectively). For discrimination between flavonoids in peel and flesh tissues, the outer layer of

approximately 2 mm thick (i.e. cuticula, epidermal layer plus some sub-epidermal tissue) is separated from the fruit using a scalpel and classified as peel. The jelly and seeds are then removed and the remainder of the fruit is classified as flesh tissue. After separation, tissues are quickly cut into pieces and frozen in liquid nitrogen before being ground into a fine powder using a pre-cooled coffee grinder. Peel and flesh tissues are lyophilised for 24 hr and then stored under desiccating conditions at 4°C until use.

3.1.2 Extraction of flavonoids from tomato tissues

Determination of flavonoid glycosides and narichalcone (2',4',6',4-tetrahydroxychalcone) in tomato fruit is carried out using a non-hydrolysing method as follows: 40 mg of freeze-dried tomato tissue is weighed and transferred to a 10 ml Pyrex glass tube. To each tube 4 ml of 75% aqueous methanol acidified with HCl to pH 2 is added. The tubes are closed with screw tops containing a Teflon inlay and incubated at room temperature (20-25°C) for 1 hr with continuous mixing on a roller band.

3.1.3 High Performance Liquid Chromatography (HPLC) conditions for flavonoid analysis

1 ml of each tomato fruit extract is taken using a disposable syringe and filtered through a 0.2 µm PTFE disposable filter (Inacom Instruments BV, The Netherlands) before injection into the HPLC system.

The HPLC system consisted of a Waters 600E Multisolvent Delivery System (Waters Chromatography), a Promis autoinjector (Separations Analytical Instruments BV) with a fixed 10 µl loop, and a Nova-Pak C_{18} (3.9 x 150 mm, particle size 4 µm) analytical column (Waters Chromatography) protected by a Guard-Pak Nova-Pak C18 insert. Both columns are placed in a LKB 2155 HPLC column oven (Pharmacia Biotech) set at 30°C. A photodiode array detector (Waters 996) is

used to record spectra of compounds eluting from the column on-line. The detector is set at recording absorbance spectra from 240 to 600 nm with a resolution of 4.8 nm, at a time interval of 1 sec. Millennium 2010 Chromatography Manager (Waters Chromatography BV) is used to control the solvent delivery system and the photodiode array detector.

HPLC separation of flavonoids in non-hydrolysed extracts is performed using a gradient of acetonitril in 0.1% TFA, at a flow rate of 1 ml/min: 12.5-17.5% linear in 3 min, then 17.5-25% in 32 min and 25-50% in 2 min, followed by a 3 min washing with 50% acetonitril in 0.1% TFA. After washing, the eluent composition is brought to the initial condition in 2 min, and the column is equilibrated for 6 min before the next injection.

HPLC data are analysed using the software of the Millennium 2010 Chromatography Manager. Absorbance spectra (corrected for baseline spectrum) and retention times of eluting peaks (with peak purity better than purity threshold value) are compared with those of commercially available flavonoid standards. Quercetin and kaempferol glycosides and narichalcone are quantified based on absorption at 360 nm. Dose-response curves (0 to 20 µg/ml) were established to quantify these compounds in the non-hydrolysed tomato extracts. Flavonoid levels in tomatoes are calculated on a dry weight basis for peel and flesh tissues. With the HPLC system and software used, the lowest detection limit for flavonoids in tomato extracts is about 0.1 µg/ml, corresponding with 10 mg/kg dry weight and 1 mg/kg fresh weight. Variation between replicate injections is generally less than 5%.

3.1.4 Characterisation of the flavonoid content in ripening tomato fruit

Two dominant flavonoids are detected in the peel of ripe tomato fruit, the flavonol rutin (quercetin 3-rutinoside) and narichalcone, which are identified by their retention time (RT) and absorbance spectrum. At least four other flavonol glycosides

are also identified in the tomato peel extracts, albeit in much smaller quantities than rutin or narichalcone. A full identification of these minor flavonol glycoside species is described in Example 8.

In contrast, the flesh tissue from ripe tomato fruit contains only traces of rutin, no other flavonoid species are detectable.

The levels of rutin and narichalcone in the peel during ripening of tomato fruit are shown in Figure 1. Rutin levels increase during tomato ripening reaching their highest levels in the over-ripe stage (approximately 1 mg/g dry weight of peel). Narichalcone is absent in the peel of green fruit but increases sharply during coloring of the fruit, reaching levels of approximately 10 mg/g dry weight in peel of turning fruit before declining through the red and over-ripe stages. The enzyme chalcone isomerase (CHI) is believed to be responsible for catalysing the formation of naringenin from narichalcone in the flavonoid biosynthetic pathway (figure 10). Applicants are of the opinion that the accumulation of narichalcone suggests that in the peel of ripening tomatoes CHI represents a rate limiting enzyme in the formation of flavonols.

3.2 Northern Analysis of ripening tomato fruit

Northern analysis is used to determine the endogenous expression of the flavonoid biosynthetic genes *chs*, *chi* and *fls* during the development of FM6203 tomato fruit.

RNA is isolated from the peel and flesh of green, breaker, turning and red fruit and also young leaves according to the protocol of van Tunen (1988). For RNA gel blot analysis, 10 µg of RNA is loaded on formaldehyde agarose gels and electrophoresed overnight at 25V. Separated RNA is then blotted overnight onto Hybond N^+ membrane (Amersham).

Petunia hybrida cDNA fragments encoding the following flavonoid biosynthetic enzymes are used as probes: chalcone synthase (CHS-A), chalcone isomerase (CHI) and flavonol synthase (FLS). These fragments are obtained by RT-PCR on RNA extracted from closed flowers of *Petunia hybrida* W115 with primer combinations F15/F16 (*chi-a*), F13/F14 (*chs-a*) and F20/F21 (*fls*). The obtained PCR products are checked by sequence analysis.

Probes are labelled with ^{32}P and purified according to methods given in Gibco Life Technologies RadPrime Labelling system. Blots are hybridised overnight at 55°C and washed three times in 2 x SSC, 0.1% SDS, 55°C, 30 min, before being exposed to X-ray film for 48 hr.

The results of the northern blot are shown in Figure 2. Both the *chs* and *fls* transcripts are abundantly present in the peel of tomato fruit in all developmental stages tested. The level of these two transcripts peaks during the breaker and turning stage of development and subsequently decreased in the red stage. The *chi* transcript level is very low in the peel of all developmental stages. Without wishing to be bound by any theory, applicants believe that this is indicative that one of the rate limiting steps in flavonoid biosynthesis in the peel may lie at the level of *chi* gene expression. This result is in agreement with the observation that narichalcone (the substrate for CHI), accumulated to high levels in breaker and turning stage fruit (Example 3.1).

In the flesh of tomato fruit, the levels of *chs*, *chi* and *fls* transcripts are very low, in agreement with the HPLC data which showed only trace amounts of rutin in this tissue (Example 3.1).

Chs, *chi* and *fls* transcripts are present in low but detectable levels in tomato leaves.

Example 4: Gene constructs.

4.1 Strategy to overexpress a rate-liming step of flavonol production in tomato fruit

During the early stages of ripening of tomato fruit narichalcone accumulates in the peel of the fruit (Example 3.1). The enzyme responsible for converting narichalcone into naringenin is CHI. The expression levels of the gene encoding CHI remain low throughout ripening of the fruit (Example 3.2) suggesting CHI may constitute a rate-liming step in the production of flavonols in the peel of tomato fruit.

The strategy consists of increasing the production of flavonols in tomato fruit by overexpression of the *Petunia hybrida* gene encoding CHI. The introduced gene is expressed under the control of the constitutive enhanced CaMV 35s promoter (also called double P35s or Pd35S).

4.1 Cloning the *chi* gene from *Petunia hybrida*

The *chi-a* gene is amplified from plasmid pMIP41, which contains the complete *chi-a* cDNA from *Petunia hybrida* inbred line V30 (Van Tunen et al. 1988), with primer combination F15/F16. These primers contain a 5' extension with a unique *Bam*HI (F15) and *Sal*I (F16) restriction site (Table 1). This results in a 0.73 kb *chi-a* fragment.

4.2 Construction of the *chi* gene fusion

The Pd35S-*chi*-Tnos gene construct is made as follows. Plasmid pFLAP10, a pUC-derivative containing a fusion of the consitutive enhanced CaMV *35s* promoter (*P35s*), the maize *c1* gene (*c1*) and the *Agrobacterium tumefaciens nos* terminator (*Tnos*), is used as recipient of the *chi-a* gene (Figure 3a). A description of the properties of plasmid pFLAP10 is given in Figure 3. The amplified *chi-a* cDNA is digested with *Bam*HI/*Sal*I and the resulting 730 bp fragment is ligated in plasmid pFLAP10 digested with the same

enzymes, thus replacing the *c1* gene with the *chi-a* gene. The resulting plasmid is denoted pFLAP50 (Figure 3b).

4.2.1 Construction of pFLAP10

The C_1 gene fusion was cloned in plasmid pUCM2, a derivative of plasmid pUCAP (Van Engelen et al. 1995, Transgenic Research 4, p. 288-290), in which the multiple-cloning-site was altered (Figure 12), in three major steps. Said altered multiple cloning site is shown in Figure 14.

Firstly, T*nos* was amplified by PCR from pBI121 with primers F12 and AB13 (see Table 1). The resulting 250bp product was cloned in pUCM2 as a *SalI/ClaI* fragment. This resulted in plasmid pFLAP1.

Secondly, the *c1* gene was cloned as a *BamHI/SalI* fragment upstream of T*nos* in pFlap2 as follows. The *c1* gene was transferred as a 2kb *EcoRI* fragment from plasmid pAL77 (Loyd 1992) to high-copy plasmid pBluescript SK-, resulting in plasmid pB1C1. The *c1* gene was isolated from pB1C1 as a 1.6kb *EcoRI/PacI* fragment and adapters F7F8 and F9F10 (Table 1) were ligated to each end of the fragment in order to add unique *BamHI* and *SalI* restriction sites on both ends of the gene and to destroy the *EcoRI* and *PacI* sites. The resulting *BamHI/SalI c1* fragment was cloned upstream of the nos terminator, resulting in plasmid pFLAP2.

Thirdly, P*d35s* was cloned as a *KpnI/BamHI* fragment upstream of *c1* in pFLAP2 as follows. To create a unique *BamHI* site at the 3' end of the *d35s* promoter, plasmid pMOG18 (Symans et al 1990, Biotechnology 8, p. 217-221) was digested with *EcoRV/BamHI*, thus removing the 3' part of the *d35s* promoter and the *gusA* gene. The 3' part of the *35s* promoter present in plasmid pAB80 (Bovy et al. (1995)) was ligated as a 0.2kb *EcoRV/BamHI* fragment in the pMOG18 vector, resulting in plasmid pMOG18B. To create a unique *KpnI* site at the 5' end of the *d35s* promoter plasmid pMOG18B was digested with *EcoRI*, the ends were polished with Klenow

polymerase, and a subsequent digest with *Bam*HI was done. The resulting 0.85kb blunt/*Bam*HI *d35s* promoter fragment was cloned into plasmid pBlC1 followed by digestion with *Xho*I and polished with Klenow polymerase/*Bam*HI. This resulted in plasmid pBld35S. Finally the *d35s* promoter was transferred as a *Kpn*I/*Bam*HI fragment from pBld35s to plasmid pFLAP2. This resulted in plasmid pFLAP10 (Figure 13).

4.3 Construction of binary vector pBBC3

To obtain a binary vector with suitable cloning sites to transfer the *chi* gene fusion into, plasmid pBBC3, a derivative of pGPTV-KAN (Becker *et al.* (1992)) is constructed as follows. Synthetic adapter F38F39 (Table 1) is ligated in plasmid pGPTV-KAN digested with *Eco*RI/*Hin*DIII. In this way the *gusA-Tnos* gene in pGPTV-KAN is replaced by a small multiple-cloning-site consisting of *Pac*I/*Eco*RI/*Hin*DIII/*Asc*I restriction sites (Figure 4a).

4.4 Transfer of the *chi* gene fusion into pBBC3

The *Pd35s-chi-Tnos* insert is transferred from pFLAP50 as a *Pac*I/*Asc*I fragment into binary vector pBBC3, digested with the same enzymes. The resulting binary plasmid is denoted pBBC50 (Figure 4b).

4.5 GPTV control plasmid

A GPTV-based binary plasmid (pSJ89) containing the ß-glucuronidase gene (with the *st-ls1* intron; Vancanneyt *et al.* 1990) under control of the CaMV *35s* promoter and the *nos* poly(A) signal (*P35s-gusA-Tnos*) is used as a control plasmid to transform FM6203 (Figure 4c). This allows direct comparison between *gus* transformed control plants and plants containing the *chi* construct as both sets of plants have gone through the tissue culture procedure.

Plasmid pSJ89 is constructed as follows: the CaMV *35s* promoter - *gus-int* fragment (Vancanneyt et al, 1990) is cloned as a *Hin*dIII - *Sac*I fragment into the same sites of plasmid pSJ34, a derivative of the binary vector pGPTV-KAN (Becker et al, 1992) in which the *Bam*HI site between the NPTII selectable marker and the gene 7 poly(A) signal is destroyed by filling in with klenow polymerase.

Example 5: Stable transformation of *chi* construct into tomato line FM6203.

5.1 *Agrobacterium tumefaciens* transformations

Binary plasmids pBBC50 and pSJ89 are introduced into *Agrobacterium* strain LBA4404 by adding 1 µg of plasmid DNA to 100 µl of competent *Agrobacterium* cells, prepared by inoculating a 50 ml culture in YEP medium (Sambrook, 1989) and growing at 28°C until the culture reaches an OD_{600} of 0.5-1.0. The cells are then pelleted, resuspended in 1 ml of $CaCl_2$ solution and dispensed into 100 µl aliquots. The DNA-*Agrobacterium* mixture is frozen in liquid nitrogen and thawed in a water bath at 37°C. After the addition of 1 ml YEP medium the bacteria are incubated at 28°C for 4 hours with gentle shaking. Finally transformed bacteria are selected on YEP-agar plates containing 50 µg/ml kanamycin. The presence of the plasmids is tested by PCR analysis using pBBC50 (chi 5 and nos ant) or pSJ89 (300 35s and gus 2) specific primers respectively (Table 1).

5.2 Tomato transformations

Seeds from tomato line FM6203 are sterilised by a 2h incubation in 1.5% hypochlorite, followed by three rinses of sterile water. The seeds are germinated and seedlings are grown for 8 days on a 1:1 mixture of vermacolite and MS medium (Murashige and Skoog, 1962; Duchefa) supplemented with 0.3% (w/v) sucrose, with a photoperiod of 16 h (3000 lux) at 25°C.

Eight-day old cotyledons are cut into 25 mm^2 squares and preincubated for 24h on tobacco suspension feeder layer plates at low light intensity (1000 lux). The tobacco leaf suspension culture is grown on plates containing MS medium including vitamins, supplemented with sucrose (3% w/v), agarose (6 g/l), 2,4-dichlorophenoxyacetic acid (2,4-D; 0.5 mg/l) and benzylaminopurine (BAP; 0.5 mg/l).

A single colony from the *Agrobacterium* LBA4404 cultures containing one of the binary vectors mentioned in Example 4.4 and 4.5 is grown for 48h in liquid Minimal A medium (Sambrook, 1989) supplemented with 50 µg/ml kanamycin to an OD_{600} of 0.5-1.0. The bacteria are pelleted by centrifugation and resuspended in MS medium including vitamins (Duchefa) and 3% (w/v) sucrose at an OD_{600} of 0.5. The cotyledon explants are incubated in the *Agrobacterium* suspension for 30 min, blotted dry on filter paper and co-cultivated for 48h on tobacco feeder layer plates at 25°C and low light intensity.

After co-cultivation, the explants are transferred to regeneration medium, consisting of MS medium supplemented with Nitsch vitamins, sucrose (2% w/v), agargel (5 g/l), zeatin-riboside (2 mg/l), kanamycin (100 mg/l) and cefotaxime (500 mg/l). Regenerating explants are transferred to fresh medium every two weeks. Regenerating kanamycin resistant shoots were transferred to rooting medium, consisting of MS medium plus B5 vitamins, supplemented with sucrose (0.5% w/v), gelrite (2 g/l), kanamycin (50 mg/l) and cefotaxime (250 mg/l). During regeneration and rooting explants are incubated in a growth chamber at 25°C with a 16h photoperiod (3000 lux). After root formation, the presence of the CHI insert is confirmed by PCR analysis of cotyledon tissue using specific primers (chi 5 and nos ant), and the presence of the GUS insert using 300 35s and gus2 specific primers (Table 1). PCR positive plantlets are transferred to soil and grown in the greenhouse.

Transgenic plants carrying the construct pBBC50 are numbered from C6 onward. Control transgenic plants carrying the construct pSJ89 are numbered from G2 onward.

Example 6: Southern analysis of transgenic plants.

The presence and the copy number of the transgenes is determined in transgenic plants by southern hybridisation. Genomic DNA is isolated from young leaves as described by Fulton *et al.*, (1995). Aliquots of 10 µg genomic DNA are digested for 16h with *EcoR*I and separated on a 0.8% TAE agarose gel. The DNA is denatured in 0.5 M NaOH, 1.5M NaCl for 45 min before being transferred to a Hybond N+ membrane (Amersham) in 20 X SSC.

The blots are probed with a 700 base pair ^{32}P radiolabeled *npt*II-specific PCR fragment, amplified from plasmid pBBC3 with primers npt IIa and npt 11b (Table 1), under stringent conditions (65°C). Prehybridisation is carried out for 2h at 65°C in a mix of 0.5 M Na_2PO_4 pH 7.2, 7% SDS. and 0.1 mg/ml denatured herring sperm DNA. Hybridisation is performed by adding denatured probe DNA to the prehybridisation medium and continuing the incubation at 65°C for 16h. The hybridised blots are washed once for 30' at 25°C in 2 x SSC, 0.1% SDS and then once for 30' at 65°C in 2 x SSC, 0.1% SDS before being autoradiographed.

The result of the southern analysis is shown in Figure 5. The control is an untransformed FM6203 plant. Southern analysis confirms the initial screening of transgenics by PCR (see Example 5.2), every pBBC50 transformed plant hybridised with the npt II probe. Transgenic plants contain either 1 or 2 copies of the insert.

Example 7: Measurement of flavonoids in transformed tomato plants.

7.1 Growth and harvest of tomato fruits

Transgenic tomato plants are grown in 10 l pots in a glasshouse at standard growth conditions (day/night temperatures 23°C/18°C, 16 hr light)). Fruits are harvested between 15-21 days post-breaker stage (corresponding to fully red ripe fruit). For discrimination between flavonoids in peel and flesh tissues, the outer layer of approximately 2 mm thick (i.e. cuticula, epidermal layer plus some sub-epidermal tissue) is separated from the fruit using a scalpel and classified as peel. The jelly and seeds are then removed and the remainder of the fruit was classified as flesh tissue. After separation, tissues are quickly cut into pieces, frozen in liquid nitrogen and stored at -80°C until use.

7.2 Extraction of flavonoids from tomato tissues

Flavonoids are determined as aglycons or as their glycosides by preparing hydrolysed and non-hydrolysed extracts, respectively.

Acid hydrolysis is used as an initial screen of transformants in order to identify those lines containing high amounts of flavonols as compared to the control. Acid hydrolysis ensures that flavonoid glycosides such as rutin and kaempferol rutinoside are hydrolysed to their respective aglycones i.e. quercetin and kaempferol.

Preparation of hydrolysed extracts is performed according to Hertog et al (1992) with some modifications. Frozen tissues are ground into a fine powder using a pre-cooled coffee grinder. Peel and flesh tissues are lyophilised for 24 h before flavonoid extraction. 50 mg of this freeze-dried material was weighed and transferred to a 6 ml Pyrex glass tube. To each tube 1.6 ml of 62.5% methanol (HPLC grade) in distilled water and 0.4 ml of 6 M HCl are added. The tubes are closed with screw caps containing a Teflon inlay and incubated for 60 min at 90°C in a waterbath.

After hydrolysis, the tubes are cooled on ice, the extracts are diluted with 2 ml of 100% methanol and sonicated for 5 min. 1 ml of the sample was then filtered over a 0.2 µm PTFE disposable filter into a standard 1.8 ml HPLC vial.

Preparation of non-hydrolysed extracts is performed as follows: Frozen tissues are ground to a fine powder using a pre-cooled coffee grinder. Peel and flesh tissues are lyophilised for 24 h before flavonoid extraction. 50 mg of freeze dried material is weighed and transferred to a 6 ml Pyrex glass tube. 4 ml of 70% methanol (HPLC grade) in distilled water is added to each tube. The tubes are closed with screw top caps containing a Teflon inlay and placed in a sonicating water bath at room temperature for 30 min. After sonication 1 ml of the sample is filtered over a 0.2 µm PTFE disposable filter into a standard 1.8 ml HPLC vial.

7.3 HPLC conditions for flavonoid analysis

Chromatography of samples is performed using a chromatography station equipped with a dual pump system and automated gradient controller (model 1100; Hewlett Packard), a Waters auto-injector (model 717) with a variable 20 µl loop and a Nova-Pak C_{18} (3.9 x 150 mm, particle size 4 µm) analytical column (Waters Chromatography) protected by a Guard-Pak Nova-Pak C18 insert. Both columns are placed in a LKB 2155 HPLC column oven (Pharmacia Biotech) set at 30°C. A photodiode array detector (model 1040M, Hewlett Packard) is used to record spectra of compounds eluting from the column on-line. The detector is set at recording absorbance spectra from 240 to 600 nm with a resolution of 4.8 nm, at a time interval of 1 second. Peak purity, identification and integration were carried out on Hewlett Packard Chemstations software version A.04.02.

HPLC separation of flavonoids present in hydrolyzed extracts (flavonols and naringenin) is carried out under isocratic conditions of 25% acetonitril (for HPLC far UV) in 0.1% trifluoroacetic acid (TFA) at a flow rate of 0.9 ml/min.

HPLC separation of flavonoids in non-hydrolysed extracts (flavonoid-glycosides and narichalcone) is performed using a gradient of acetonitril in 0.1% TFA, at a flow rate of 1.0 ml/min: 5-25% linear in 30 min, then 25-30% in 5 min and 30-50% in 2 min followed by a 3 min washing with 50% acetonitril in 0.1% TFA. After washing, the eluent composition is brought to the initial condition in 2 min, and the column is equilibrated for 6 min before next injection.

HPLC data are analysed using the software of the Hewlett Packard Chemstations software version A.04.02. Absorbance spectra (corrected for baseline spectrum) and retention times of eluting peaks (with peak purity better than purity threshold value) are compared with those of commercially available flavonoid standards. Quercetin and kaempferol aglycons are detected and calculated from their absorbance at 370 nm, naringenin at 280 nm and flavonol-glycosides as well as narichalcone at 360 nm. Flavonoid levels in tomatoes are calculated on a dry weight basis. With the HPLC system and software used, the lowest detection limit for flavonoids in tomato extracts is about 0.1 µg/ml, corresponding with 10 mg/kg dry weight and 1 mg/kg fresh weight.

Using flavonoid standards (obtained from Apin Chemicals Ltd, Abingdon, UK) it is established that during the hydrolysis step, aglycons are released from their respective glycosides for 100%, while chemically converted into naringenin for more than 95%. Recoveries of quercetin, kaempferol and naringenin standards added to peel or flesh extracts just before hydrolysis are more than 90%.

Example 8: Characterisation of the flavonoid content in transgenic tomato fruit.

8.1 Flavonoids in peel and flesh of control tomatoes

In hydrolysed extracts of control red fruit of variety FM6203 transformed with pSJ89, both quercetin and kaempferol are present in peel tissue (Figure 6a). In contrast, the hydrolysed extracts of flesh tissue from this fruit contain only traces of quercetin with no detectable levels of kaempferol (Figure 6b). Without wishing to be bound by any theory, applicants believe that the small amount of quercetin detected in the hydrolysed extracts of flesh originates from the vascular tissue in the flesh. Chromatograms obtained at 280 nm (not shown) of the same extracts reveal a large peak of naringenin in the peel, but not in the flesh. There is no significant difference in the identity and quantity of flavonoids found in the control pSJ89-transformed tomatoes and those found in untransformed tomatoes (data not shown).

In non-hydrolysed extracts of control tomatoes transformed with pSJ89, at least 5 different flavonol-glycosides as well as narichalcone are detected in the peel (Figure 7). NMR-studies (not shown) prove that the peak at RT = 16.9 min is rutin while the peak at 15.2 min is a quercetin-3-trisaccharide: rutin with apiose linked to the glucose of the rutinoside. The retention time and absorbance spectrum of the minor peak at 17.3 min correspond with those of quercetin-3-glucoside, while those of the peak at 19.7 min correspond with kaempferol-3-rutinoside. The small peak at 20.4 min has an absorbance spectrum comparable to kaempferol-3-rutinoside, but its higher RT value indicates a yet unknown kaempferol-glycoside. The large peak at 33.2 min is narichalcone. Aglycons of quercetin and kaempferol, as well as naringenin (all present in hydrolysed peel extracts) are not detectable in any of the non-hydrolysed extracts. In the non-hydrolysed flesh sample only a small peak corresponding to rutin is detected (data not shown).

After comparing the flavonoid species in hydrolysed extracts with those in non-hydrolysed extracts of the same tissue, we conclude that the presence of quercetin and kaempferol aglycons in the hydrolysed extracts results from hydrolysis of their respective glycosides; the presence of naringenin in hydrolysed peel extracts results from isomerization of narichalcone during the hydrolysis step (cf. Example 7.3).

8.2 Flavonoids in fruits of transformed tomato plants

To determine whether the pBBC50 construct was able to overcome the suspected rate limiting step in flavonol production in tomato fruit, transformants are analysed for the presence of flavonoids in the flesh and peel of their fruits. This screening is performed by HPLC using hydrolysed extracts. Thirty six independent plants transformed with pBBC50, as well as six control plants transformed with pSJ89 are analysed.

Analysis of hydrolysed extracts of flesh samples from pBBC50 transformed fruit reveals no significant increase in flavonoids compared to the control pSJ89 fruit (Figure 8). The differences in quercetin concentration in tomato flesh that are shown in Figure 8 are believed to be within the experimental error. Said experimental error is believed to be relatively high when working at low concentration near the detection limit of 20 µg/g DW flesh.

In contrast, analysis of the hydrolysed extracts of peel of the tomato fruit reveals that the presence of the pBBC50 construct results in a significant increase in the levels of both quercetin and kaempferol type flavonols in a proportion of transformed plants (Table 2, figure 11). Hydrolysed extracts of pBBC50 transformed plants display a range of peel quercetin concentrations with one line expressing a 69 fold increase over the pSJ89 transformed control lines (plant C20). The amount of kaempferol present in the hydrolysed extracts of pBBC50 transformed plants correlates with their quercetin concentrations

- lines with higher concentrations of quercetin seem also to possess higher concentrations of kaempferol in hydrolysed extracts of their peel. Applicants wish to point out that the variety in concentrations of quercetin, kaempferol and naringenin as measured for the transformed plants is believed to represent the common representation of a transgenic population. Applicants however wish to stress that the currently obtained data clearly show an increase in the level of quercetin and kaempferol in the peel of transformed plants.

The pBBC50 transformed plants also display a range of peel naringenin concentrations (note that these measurements were carried out on hydrolysed extracts, therefore the naringenin was originally derived from narichalcone as can be deduced from analysis of non-hydrolysed extracts cf. Example 8.1). In general, those transformants possessing increased concentrations of flavonols in their peel also possess decreased concentrations of naringenin when compared to the control fruit (Table 2). That the decrease in naringenin concentrations correlates with an increase in flavonol concentrations in the peel of the pBBC50 transformed fruits strongly suggests that CHI no longer represents a rate-limiting step in these plants.

The significant increase in fruit flavonol levels seen in the pBBC50 transformed plants seems to reveal that, as suggested in Example 2, CHI represents a major rate limiting step in the production of flavonols in tomato peel which has now been overcome by the heterologous expression of the petunia *chi* gene.

Using non-hydrolysed extracts, subsequently it is analysed in which form the flavonoids accumulated in the tomato peel of pBBC50 transformed plants. Figure 9 shows an example of HPLC chromatograms obtained with non-hydrolysed peel extracts from a pBBC50 transformed tomato. As with the control FM6203 peel, rutin (RT= 16.0 min) represents the major quercetin glycoside which accumulates in the peel of the pBBC50 transformed tomato. In addition, significant amounts of isoquercitrin (quercetin 3-

glucoside) (RT = 17.4 min) also accumulate in the pBBC50 peel. The peak at 19.7 min appeared to contain a mixture of two compounds: the retention time and absorbance spectra of the major component corresponded to that of kaempferol rutinoside, whilst that of the minor component has an absorbance spectrum comparable to that of a quercetin glycoside. The small peak at 20.3 min has an absorbance spectrum comparable to kaempferol-3-rutinoside, but its higher RT value indicates a yet unknown kaempferol-glycoside.

Quercetin and kaempferol aglycons, all clearly present in the hydrolysed extracts, are not detectable in the non-hydrolysed peel extracts of pBBC50 transformed tomatoes. Therefore, applicants believe that these compounds are fully derived from hydrolysis of their respective glycosides. No anthocyanins accumulate in the transformed red tomatoes, as is obvious from the absence of any peak in the chromatograms recorded at 520 nm (not shown).

Table 1. Overview of PCR primers and adapters used.

primer *	sequence (5′ to 3′)	
gus2	GCATCACGCAGTTCAACGCTG	(SeQ ID 3)
300 35S	CGCAAGACCCTTCCTCTATATAAG	(SeQ ID 4)
nos ant	CCGGCAACAGGATTCAATCTT	(SeQ ID 5)
chi5	GGTCGTGCCATTGAGAAGTT	(SeQ ID 6)
nptII a	GAGGCGATTCGGCTATGACTG	(SeQ ID 7)
npt IIb	ATCGGGAGCGGCGATACCGTA	(SeQ ID 8)
F7	AATTGCACCGGTCG	(SeQ ID 9)
F8	GATCCGACCG	(SeQ ID 10)
F9	TAGCCATGGG	(SeQ ID 11)
F10	TCGACCCATGGCTAAT	(SeQ ID 12)
F12	CCCGTCGACTTTCCCCGATCGTTCAAACATTTGGC	(SeQ ID 13)
F13	CCCGGATCCAAAAATGGTGACAGTCGAGG	(SeQ ID 14)
F14	CCGGTCGACGCAAATACATTCATGGCAAACG	(SeQ ID 15)
F15	GGCGGATCCAAAAATGTCTCCTCCAGTGTC	(SeQ ID 16)
F16	CCCGTCGACCTAAACTAGACTCCAATCACT	(SeQ ID 17)
F20	CGGGGATCCAGAGGGCCTAACTTCTGTATAGAC	(SeQ ID 18)
F21	CCCGTCGACTCGCGAAGATATAGCTAATCG	(SeQ ID 19)
F38	AATTGGGCGCGCCAAGCTTCCGAATTCTTAATTAAG	(SeQ ID 20)
F39	AGCTCTTAATTAAGAATTCGGAAGCTTGGCGCGCCC	(SeQ ID 21)
AB13	CCCATCGATGCGTCTAGTAACATAGATGAC	(SeQ ID 22)

* Adapters are made by combining two primers, heating to 95°C for 5′ and anneal both primers by cooling slowly to room temperature.

Table II. Flavonoid level in peel of transformed plants

Line number	[Quercetin] µg/g dry weight peel	[Kaempferol] µg/g dry weight peel	[Naringenin] µg/g dry weight peel
G4	115	15	280
G2	206	32	1095
G95	210	45	980
G3	253	27	523
G96	265	45	625
G97	345	55	1150
C69	115	10	105
C102	190	20	280
C41	208	28	789
C22	215	40	235
C54	230	40	410
C73	250	35	930
C6	300	15	375
C10	301	37	383
C64	320	35	470
C24	325	35	545
C53	350	65	345
C33	388	55	2107
C120	530	45	230
C51	690	217	43
C57	750	130	830
C56	1490	100	270
C48	2530	215	60
C9	3155	95	80
C34	3375	175	75
C118	4355	615	65
C25	4445	455	70
C88	4850	570	115
C86	4980	510	130
C39	5540	325	80
C65	6203	596	80
C72	6372	951	20
C49	6970	735	50
C40	7244	1147	40
C67	7405	900	80
C38	7795	735	50
C11	7995	805	135
C103	8705	840	105
C104	10055	870	110
C66	10885	1370	70
C87	13410	1250	95
C20	16520	2048	80

Legend: HPLC analysis of flavonoid aglycons in hydrolysed peel extracts of transgenic tomatoes transformed with either PSJ89 (G series) or pBBC 50 (C series)

Sequence listings

sequence ID no 1

Amino acid sequence of chalcone isomerase isolated from petunia: Source: van Tunen et al, EMBO J. 7, 1257-1263, Pir database accession number SO4725.

MSPPVSVTKMQVENYAFAPTVNPAGSTNTLFLAGAGHRGLEIEGKFVKFTAIGVYLEESA
IPFLAEKWKGKTPQELTDSVEFFRDVVTGPFEKFTRVTMILPLTGKQYSEKVAENCVAHW
KGIGTYTDDEGRAIEKFLDVFRSETFPPGASIMFTQSPLGLLTISFAKDDSVTGTANAVI
ENKQLSEAVLESIIGKHGVSPAAKCSVAERVAELLKKSYAEEASVFGKPETEKSTIPVIG V

Sequence ID no 2

Nucleotide sequence encoding for protein with chalcone isomerase activity.

Source: van Tunen et al, EMBO J. 7, 1257-1263, 1988
EMBL database accession number: X14589

atgtctcctccagtgtccgttactaa aatgcaggtt gagaattacg ctttcgcacc
gaccgtgaaccctgctggttccaccaatac cttgttcctt gctggtgctg ggcatagagg
tctggagatagaagggaagtttgttaagtt tacggcgata ggtgtgtatc tagaagagag
tgctattccttttctggccgaaaaatggaa aggcaaaacc ccccaggagt tgactgactc
ggtcgagttctttagggatgttgttacagg tccatttgag aaatttactc gagttactat
gatcttgcccttgacgggcaagcagtactc ggagaaggtg gcggagaatt gtgttgcgca
ttggaaggggataggaacgtatactgatga tgagggtcgt gccattgaga agtttctaga
tgttttccggagtgaaacttttccacctgg tgcttccatc atgtttactc aatcacccct
agggttgttgacgattagcttcgctaaaga tgattcagta actggcactg cgaatgctgt
tatagagaacaagcagttgtctgaagcagt gctggaatca ataattggga agcatggagt
ttctcctgcggcaaagtgtagtgtcgctga aagagtagcg gaactgctca aaaagagcta
tgctgaagaggcatctgtttttggaaaacc ggagaccgag aaatctacta ttccagtgat
tggagtctagttt

CLAIMS

1. A method for producing a plant capable of exhibiting altered levels of flavonoids comprising incorporating into said plant one or more gene sequences encoding a protein with chalcone isomerase activity, or incorporating a nucleotide sequence encoding a protein functionally equivalent thereto.

2. Method according to claim 1 characterised in that said gene or genes encode chalcone isomerase, isolated from a species selected from the group comprising tomato plant, petunia, maize, arabidopsis, alfalfa, pea, bean, grape, apple.

3. Method according to claim 1 characterised in that said gene or genes or nucleotide sequence encode the protein chalcone isomerase, isolated from petunia.

4. A method according to claim 1 characterised in that said plant is a tomato plant.

5. A method according to any one of claims 1 to 4 characterised in that levels of "specific flavonoids" in said plant are increased compared to untransformed plants.

6. A method according to any one of claims 1-5 characterised in that the level of "specific flavonoids" in transformed plants is at least 4 times higher than in similar untransformed plants, more preferred 5-100, most preferred 10-40 times higher than in similar untransformed plants.

7. A method according to any one of claims 1 to 6 characterised in that the level of specific flavonoids in the peel of the fruit of said plant is increased.

8. A method according to any one of claims 1 to 7 wherein said flavonoid is a flavonol.

9. A method according to any of claims 1 to 7 characterised in that the flavonoid is quercetin or kaempferol or their glycosides or any other derivative thereof.

10. A method according to any one of claims 1 to 8 characterised in that the introduced gene comprises a nucleotide sequence or complementary nucleotide sequence selected from:

(i) a nucleotide sequence, encoding an amino acid sequence having at least 40 % similarity, to seq ID No1;

(ii) a nucleotide sequence capable of hybridising under low stringent conditions to a sequence selected from the group of sequences set forth under (i) above;

(iii) a nucleotide sequence encoding a protein that is being functionally equivalent to the protein encoded by seq ID no 1.

11. A method according to claim 10, characterised in that said gene comprises a nucleotide sequence encoding an amino acid sequence having at least 60% similarity, preferably at least 90%, more preferred at least 95%, most preferred at least 95% similarity to the sequence as set forth in seq ID No1.

12. A method according to claim 10, characterised in that said gene comprises a nucleotide sequence, encoding an amino acid sequence having 99-100% similarity, to seq ID No1.

13. A method according to any of claims 1-12, characterised in that said nucleotide sequence comprises a sequence which has at least 50, more preferably at least 60% similarity, more preferred at least 75%, even more preferred at least 80%, still more preferred at least 90%, most preferred 95-100% similarity to seq ID no 2, and whereby said sequence encodes a protein having chalcone isomerase activity.

14. A method according to any one of claims 1-13 characterised in that the gene encoding a protein with chalcone isomerase is operably linked to a promoter.

15. A method according to claim 14 characterised in that the promoter is selected from the group of:

(a) constitutive promoters, such as carnation edged ring virus, cauliflower mosaic virus 35 S promoter, enhanced cauliflower mosaic virus 35 S promoter;

(b) fruit specific promoters;

(c) any other suitable promoter such as GBSS (granular bound starch synthase) promoter.

16. A method according to claim 15 characterised in that the promoter is a fruit specific promoter selected from the group of PG, 2A11, E8, E4, and fpb11.

17. A method according to any of claims 1-16 comprising the additional step increasing phenylalanine biosynthesis.

18. A plant having one or more transgenes, each encoding a protein with chalcone isomerase activity or a protein functionally equivalent thereto, incorporated into its genome such that its ability to produce flavonoids is altered.

19. A plant according to claim 18, whereby said plant is a tomato plant, prepared according to the method of any one of claims 1 to 17.

20. A DNA construct suitable for use to overexpress the encoded protein, comprising sequences coding for a protein with chalcone isomerase activity, or a functionally equivalent sequence thereof, operably linked to a promoter.

21. A plant comprising a DNA construct according to claim 20.

22. A transformed plant having enhanced levels of specific flavonoids compared to similar, untransformed plants.

23. A tomato plant having enhanced levels of specific flavonoids in the peel of the fruit compared to the level of flavonoids in the peel of the fruit of untransformed plants.

24. Seeds, fruits, progeny and hybrids of a plant according to any one of claims 18, 19, 21, 22, or 23.

25. A food product comprising at least part of a plant according to any one of claims 18, 19, 21, 22, or 23.

26. A food product according to claim 25 characterised in that the food product is sauce, dressing, ketchup or soup.

27. A skin or hair protective product comprising at least part of a plant according to any one of claims 18, 19, 21, 22, or 23.

28. A pharmaceutical product comprising at least part of a plant according to any one of claims 18, 19, 21, 22, or 23.

(63)

Fig.1A.

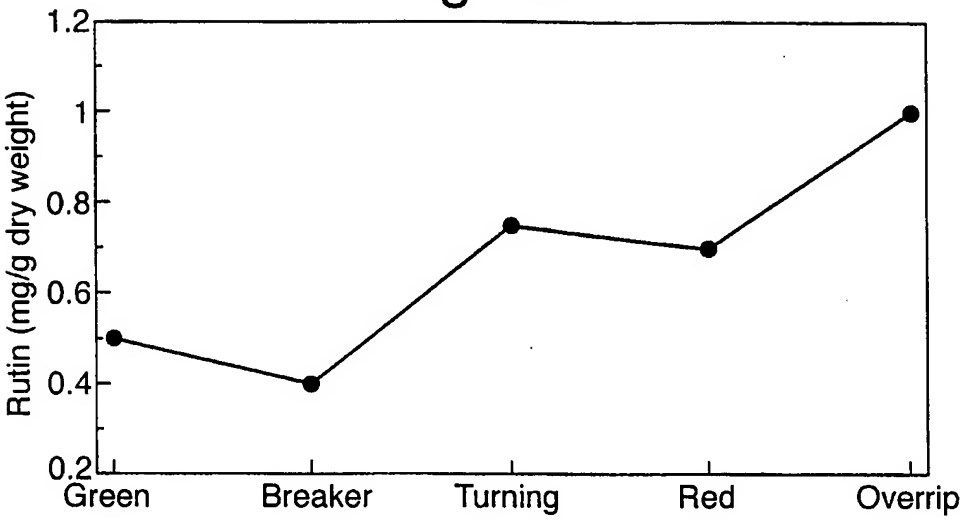


Fig.1B.

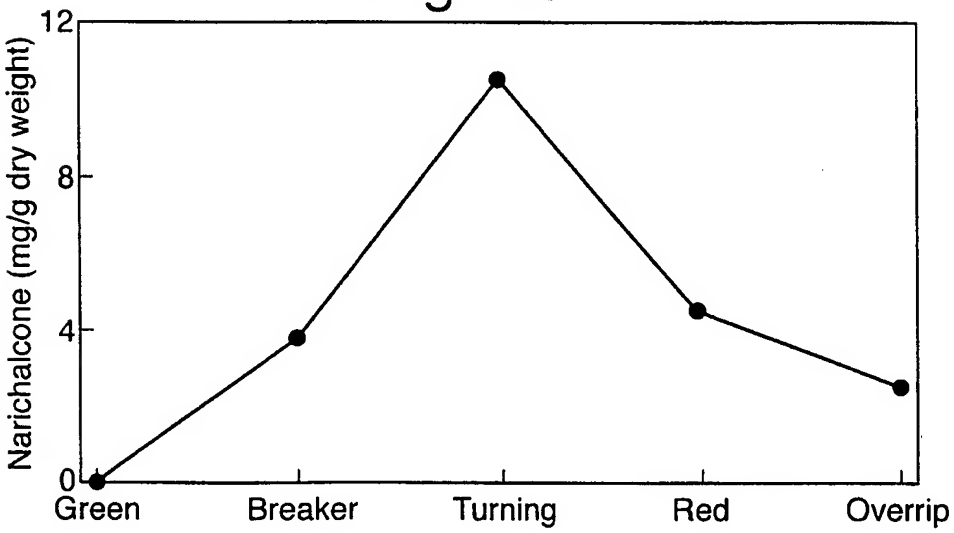


Fig.2.

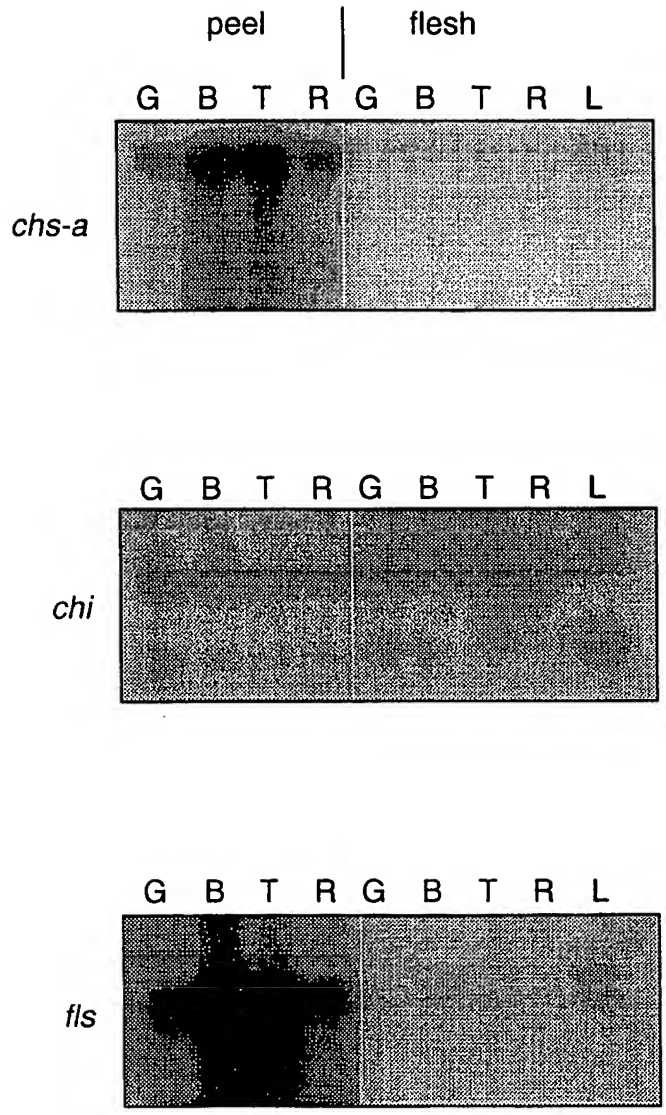


Fig.3.

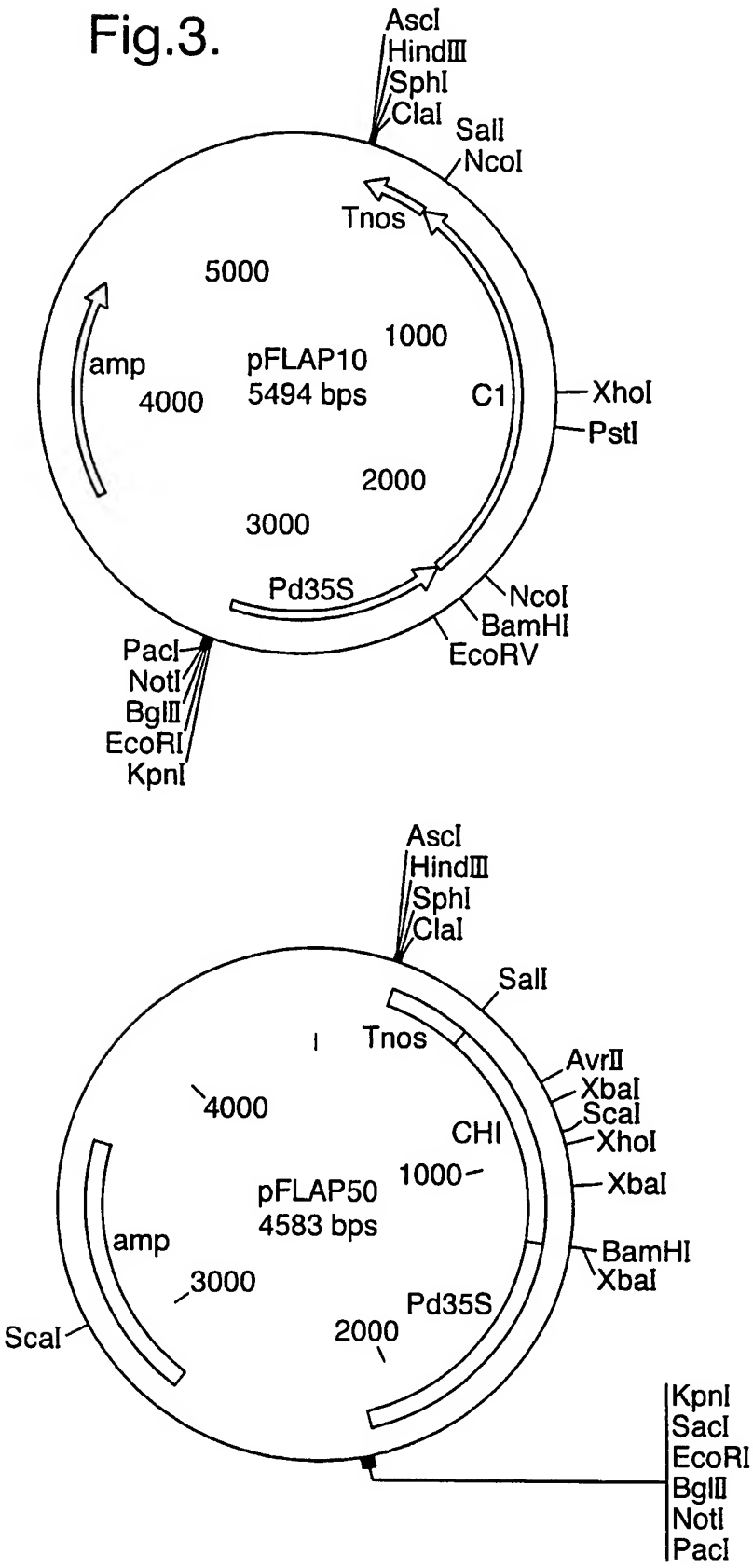
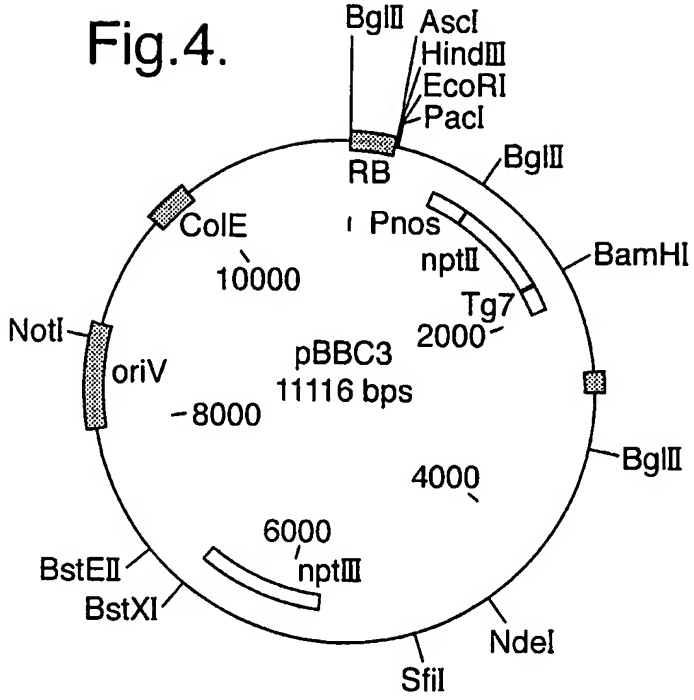

AscI
HindIII
SphI
ClaI
SalI
NcoI
Tnos
5000
1000
amp
pFLAP10
5494 bps
4000
C1
XhoI
PstI
2000
3000
Pd35S
NcoI
BamHI
EcoRV
PacI
NotI
BglII
EcoRI
KpnI
AscI
HindIII
SphI
ClaI
SalI
Tnos
AvrII
XbaI
ScaI
XhoI
4000
CHI
1000
XbaI
pFLAP50
4583 bps
amp
BamHI
XbaI
3000
Pd35S
ScaI
2000
KpnI
SacI
EcoRI
BglII
NotI
PacI

Fig.4.


BglII
AscI
HindIII
EcoRI
PacI
RB
BglII
ColE
Pnos
nptII
BamHI
10000
Tg7
2000
NotI
oriV
pBBC3
11116 bps
8000
BglII
4000
6000
nptIII
BstEII
BstXI
NdeI
SfiI

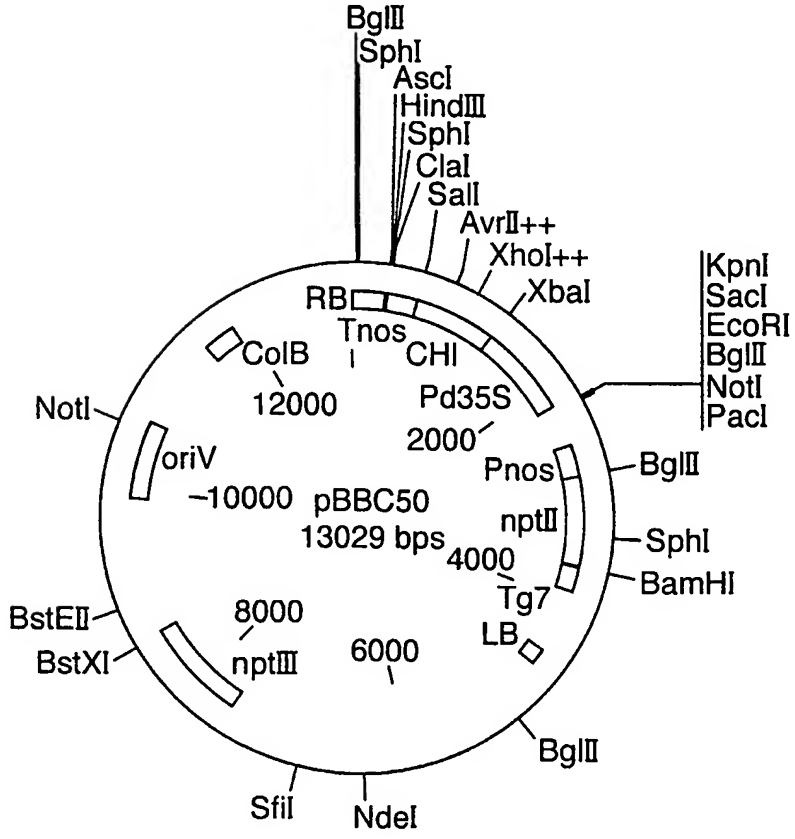

BglII
SphI
AscI
HindIII
SphI
ClaI
SalI
AvrII++
XhoI++
XbaI
KpnI
SacI
EcoRI
BglII
NotI
PacI
RB
Tnos
ColB
CHI
12000
Pd35S
NotI
2000
oriV
Pnos
BglII
10000
pBBC50
nptII
13029 bps
SphI
4000
BamHI
Tg7
8000
LB
BstEII
6000
BstXI
nptIII
BglII
SfiI
NdeI

Fig.4 (Cont).

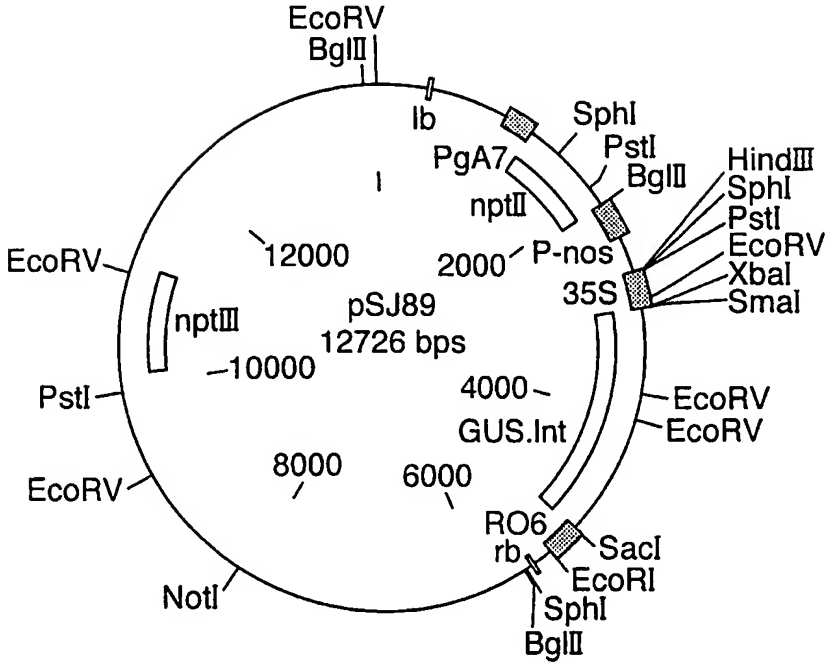

EcoRV
BglII
Ib
SphI
PstI
BglII
HindIII
SphI
PstI
EcoRV
XbaI
SmaI
PgA7
nptII
P-nos
35S
2000
12000
EcoRV
nptIII
pSJ89
12726 bps
10000
PstI
4000
GUS.Int
EcoRV
EcoRV
8000
6000
EcoRV
RO6
rb
SacI
EcoRI
SphI
BglII
NotI

Fig.5.

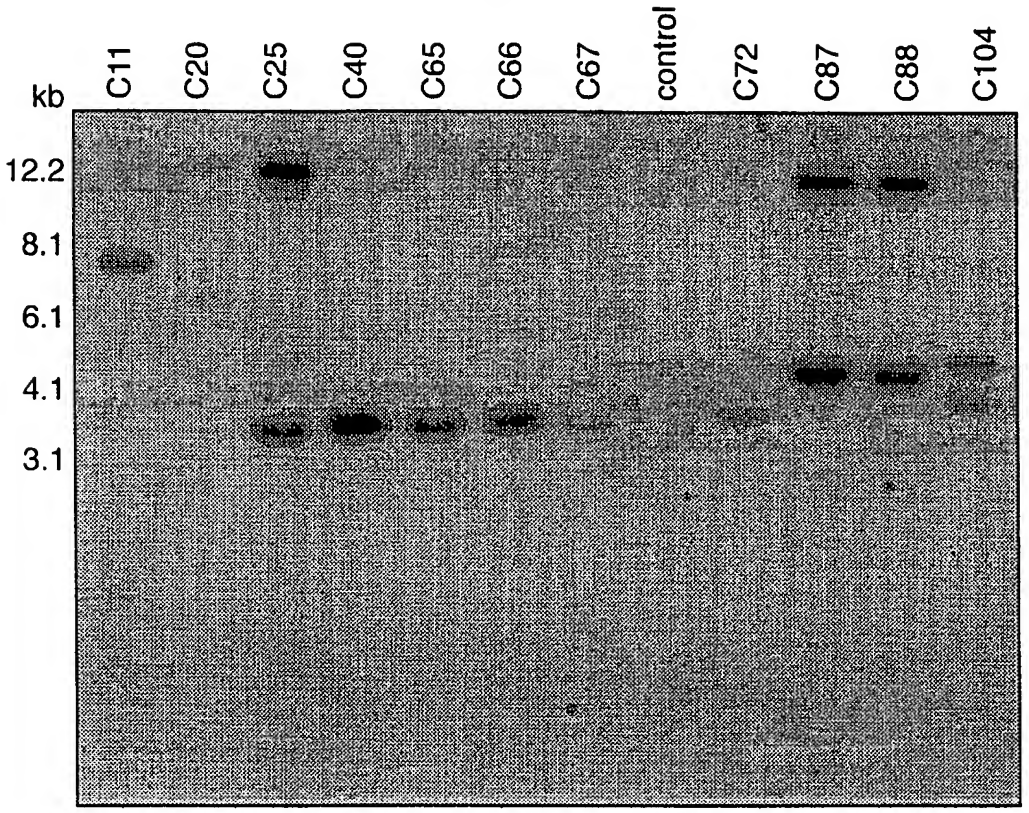

kb
C11
C20
C25
C40
C65
C66
C67
control
C72
C87
C88
C104
12.2
8.1
6.1
4.1
3.1

Fig.9.

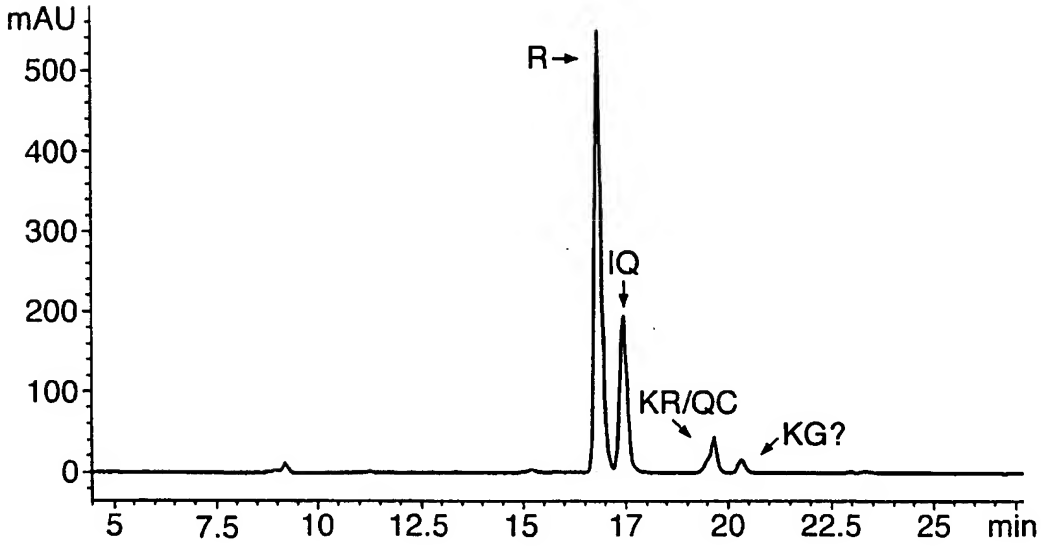

mAU
500
400
300
200
100
0
R
IQ
KR/QC
KG?
5
7.5
10
12.5
15
17
20
22.5
25
min

Fig.6A.

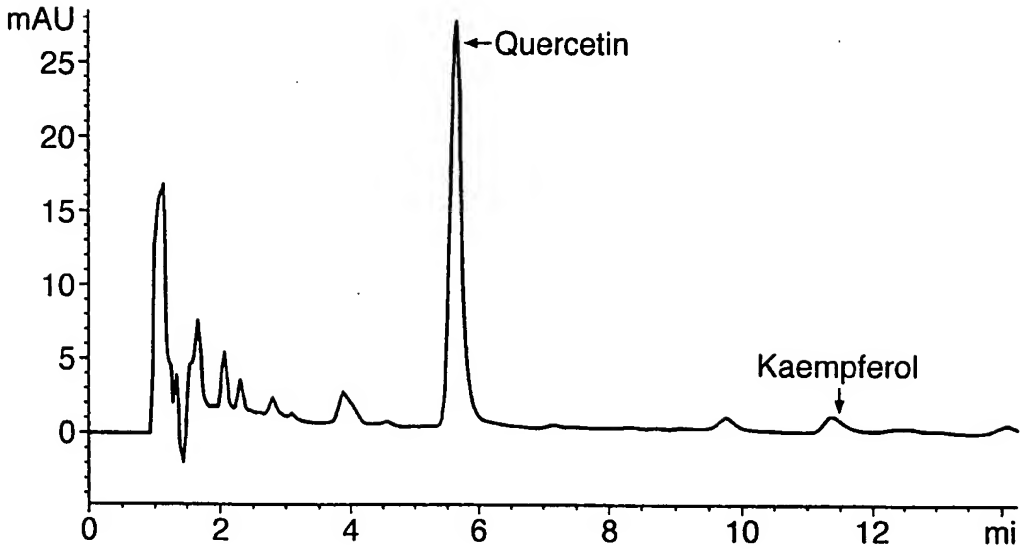

mAU
25
20
15
10
5
0
Quercetin
Kaempferol
0
2
4
6
8
10
12
mi

Fig.6B.

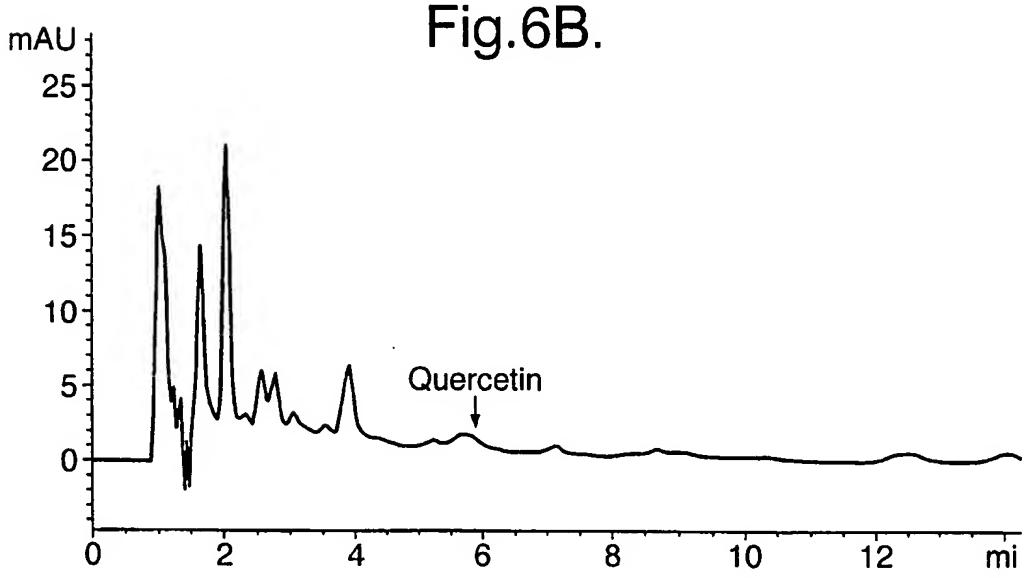

mAU
25
20
15
10
5
0
Quercetin
0
2
4
6
8
10
12
mi

Fig.7.

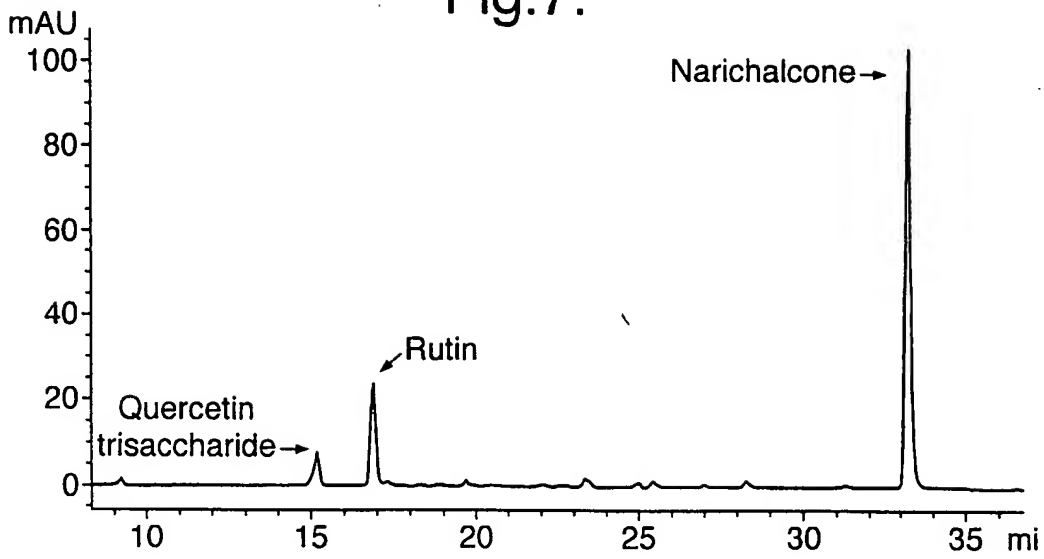

mAU
100
80
60
40
20
0
Narichalcone
Rutin
Quercetin
trisaccharide
10
15
20
25
30
35
mi

Fig.8.

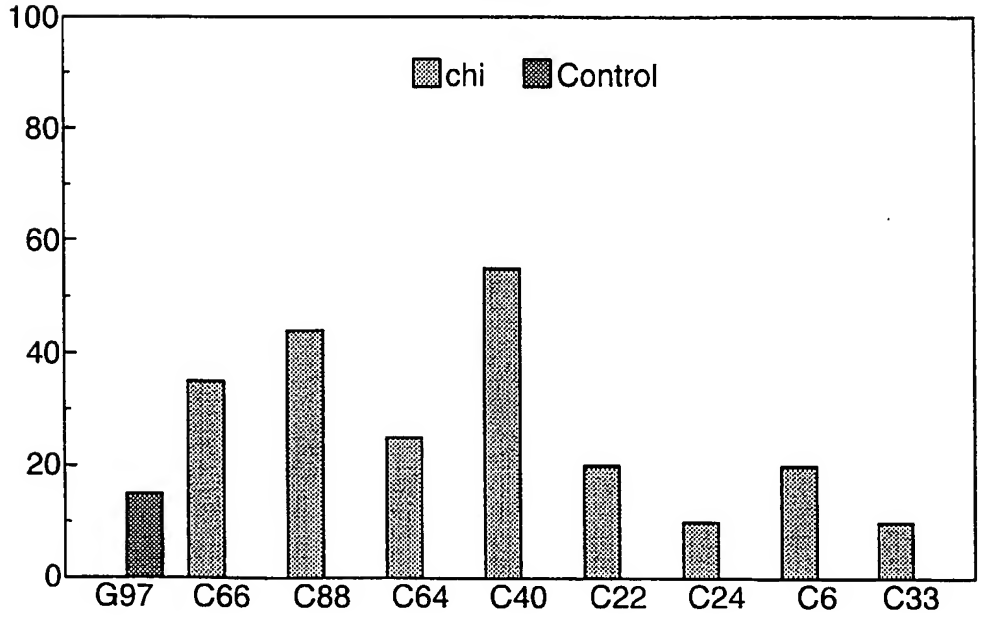

100
80
60
40
20
0
chi
Control
G97
C66
C88
C64
C40
C22
C24
C6
C33

Fig.10.

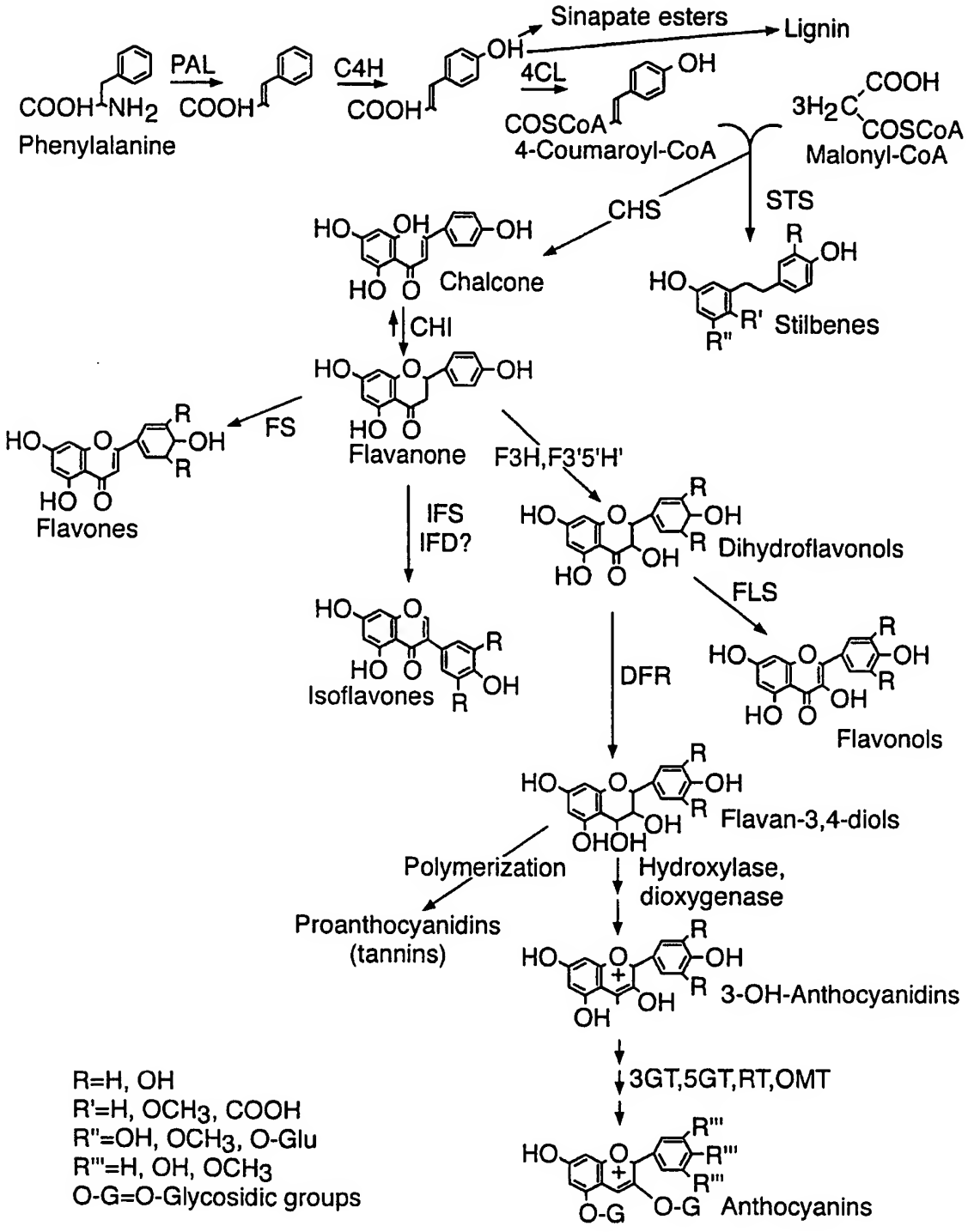

Sinapate esters
Lignin
PAL
C4H
4CL
Phenylalanine
4-Coumaroyl-CoA
Malonyl-CoA
CHS
STS
Chalcone
Stilbenes
CHI
Flavanone
FS
Flavones
F3H,F3'5'H'
IFS
IFD?
Dihydroflavonols
FLS
Isoflavones
DFR
Flavonols
Flavan-3,4-diols
Polymerization
Hydroxylase,
dioxygenase
Proanthocyanidins
(tannins)
3-OH-Anthocyanidins
3GT,5GT,RT,OMT
Anthocyanins
R=H, OH
R'=H, OCH3, COOH
R''=OH, OCH3, O-Glu
R'''=H, OH, OCH3
O-G=O-Glycosidic groups

Fig.11.

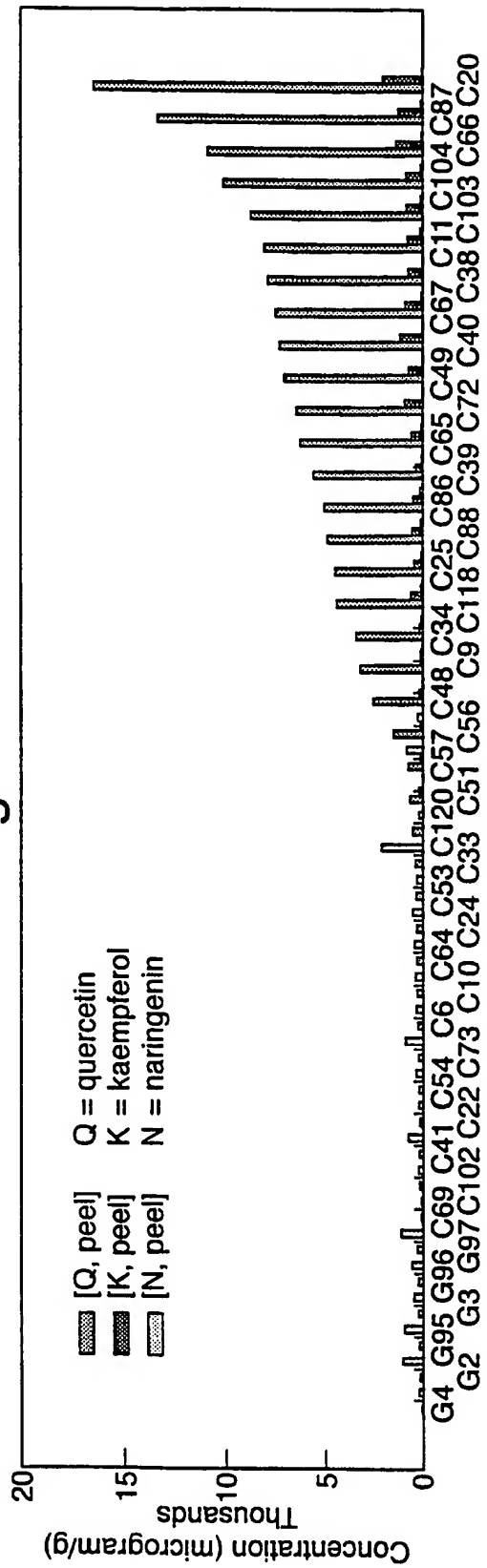
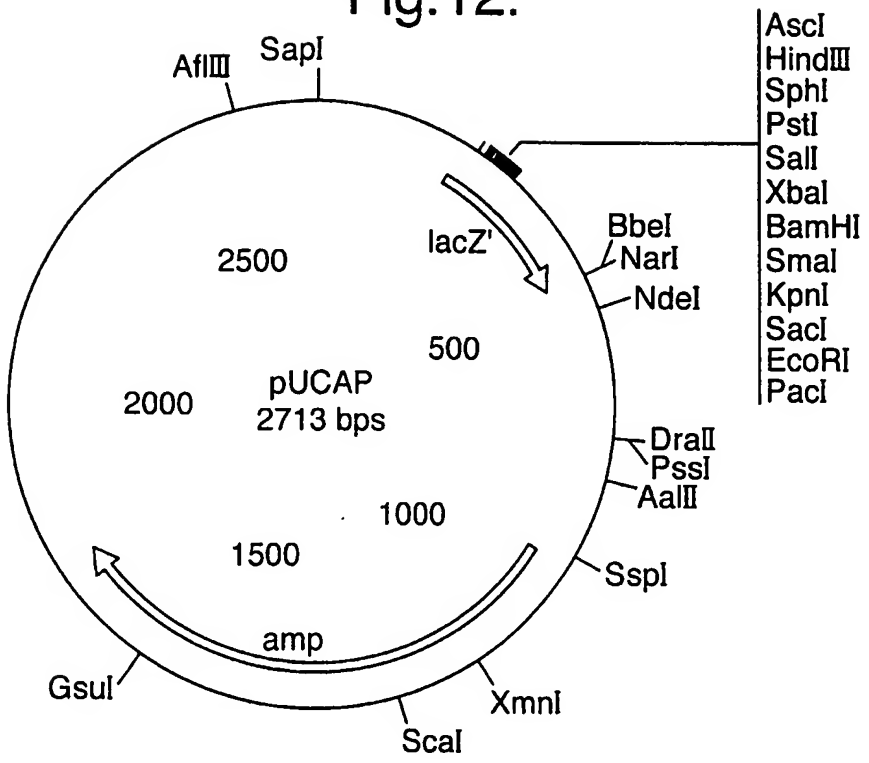

[Q, peel]
[K, peel]
[N, peel]
Q = quercetin
K = kaempferol
N = naringenin
20
15
10
5
0
Thousands
Concentration (microgram/g)
G4 G95 G96 C69 C41 C54 C6 C64 C53 C120 C57 C48 C34 C25 C86 C65 C49 C67 C11 C104 C87
G2 G3 G97 C102 C22 C73 C10 C24 C33 C51 C56 C9 C118 C88 C39 C72 C40 C38 C103 C66 C20
Line number

Fig.12.


AflIII
SapI
AscI
HindIII
SphI
PstI
SalI
XbaI
BamHI
SmaI
KpnI
SacI
EcoRI
PacI
lacZ'
BbeI
NarI
NdeI
2500
500
pUCAP
2713 bps
2000
DraII
PssI
AalII
1000
1500
SspI
amp
GsuI
XmnI
ScaI

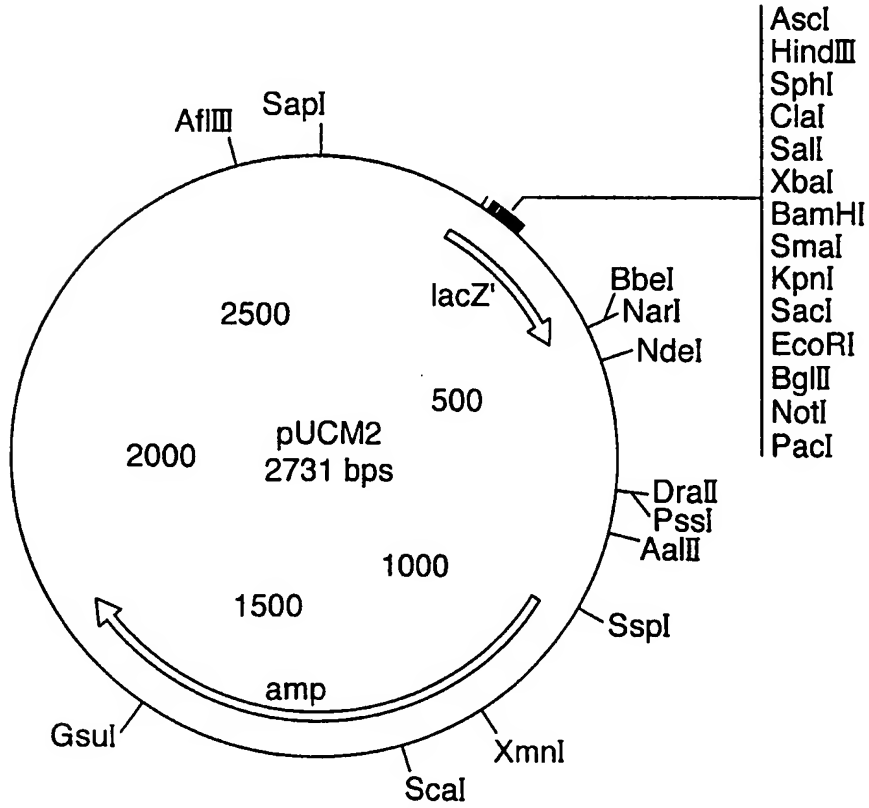

AflIII
SapI
AscI
HindIII
SphI
ClaI
SalI
XbaI
BamHI
SmaI
KpnI
SacI
EcoRI
BglII
NotI
PacI
lacZ'
BbeI
NarI
NdeI
2500
500
pUCM2
2731 bps
2000
DraII
PssI
AalII
1000
1500
SspI
amp
GsuI
XmnI
ScaI

Fig.13.

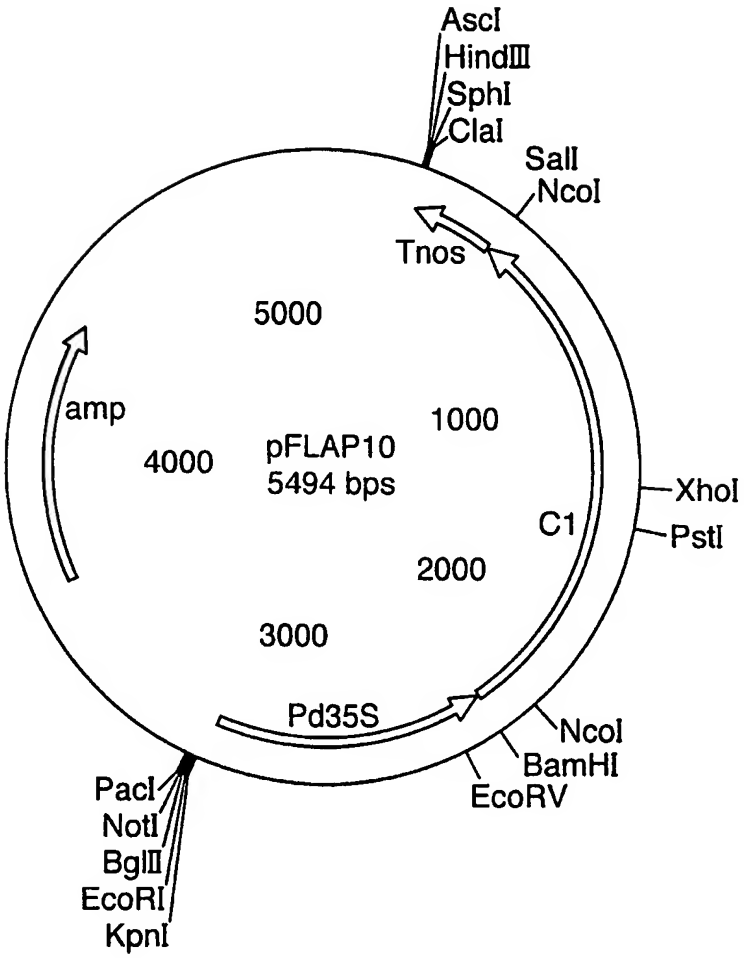


Fig.14.

GGCGCGCCAAGCTTGCATGCATCGATATGGTCGACTCTAGAGGATCCCCGGGTACCGAG
CTCGAATTCCAGATCTGCGGCCGCTTAATTAA

SEQUENCE LISTING

<110> UNILEVER PLC
UNILEVER NV

<120> METHODS AND COMPOSITION FOR MODULATING FLAVONOID CONTENT

<130> F7456

<140>
<141>

<160> 23

<170> PatentIn Ver. 2.1

<210> 1
<211> 241
<212> PRT
<213> PETUNIA HYBRIDA

<400> 1

```
Met Ser Val Ser Val Thr Lys Met Val Asn Tyr Ala Ala Thr Val Asn
  1               5                  10                  15

Ala Gly Ser Thr Asn Thr Ala Gly Ala Gly His Arg Gly Gly Lys Val
             20                  25                  30

Lys Thr Ala Gly Val Tyr Ser Ala Ala Lys Trp Lys Gly Lys Thr Thr
         35                  40                  45

Asp Ser Val Arg Asp Val Val Thr Gly Lys Thr Arg Val Thr Met Thr
     50                  55                  60

Gly Lys Tyr Ser Lys Val Ala Asn Cys Val Ala His Trp Lys Gly Gly
 65                  70                  75                  80

Thr Tyr Thr Asp Asp Gly Arg Ala Lys Asp Val Arg Ser Thr Gly Ala
                 85                  90                  95

Ser Met Thr Ser Gly Thr Ser Ala Lys Asp Asp Ser Val Thr Gly Thr
            100                 105                 110

Ala Asn Ala Val Asn Lys Ser Ala Val Ser Gly Lys His Gly Val Ser
        115                 120                 125

Ala Ala Lys Cys Ser Val Ala Arg Val Ala Lys Lys Ser Tyr Ala Ala
```

130 135 140

Ser Val Gly Lys Thr Lys Ser Thr Val Gly Val
145 150 155

<210> 2
<211> 729
<212> DNA
<213> PETUNIA HYBRIDA

<400> 2
atgtctcctc cagtgtccgt tactaaaatg caggttgaga attacgcttt cgcaccgacc 60
gtgaaccctg ctggttccac caataccttg ttccttgctg gtgctgggca tagaggtctg 120
gagatagaag ggaagtttgt taagtttacg gcgataggtg tgtatctaga agagagtgct 180
attccttttc tggccgaaaa atggaaaggc aaaaccccca aggagttgac tgactcggtc 240
gagttcttta gggatgttgt tacaggtcca tttgagaaat ttactcgagt tactatgatc 300
ttgcccttga cgggcaagca gtactcggag aaggtggcgg agaattgtgt tgcgcattgg 360
aaggggatag gaacgtatac tgatgatgag ggtcgtgcca ttgagaagtt tctagatgtt 420
ttccggagtg aaacttttcc acctggtgct tccatcatgt ttactcaatc acccctaggg 480
ttgttgacga ttagcttcgc taaagatgat tcagtaactg gcactgcgaa tgctgttata 540
gagaacaagc agttgtctga agcagtgctg gaatcaataa ttgggaagca tggagtttct 600
cctgcggcaa agtgtagtgt cgctgaaaga gtagcggaac tgctcaaaaa gagctatgct 660
gaagaggcat ctgtttttgg aaaaccggag accgagaaat ctactattcc agtgattgga 720
gtctagttt 729

<210> 3
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PRIMER

<400> 3
gcatcacgca gttcaacgct g 21

<210> 4
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PRIMER

<400> 4
cgcaagaccc ttcctctata taag 24

<210> 5
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PRIMER

<400> 5
ccggcaacag gattcaatct t 21

<210> 6
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PRIMER

<400> 6
ggtcgtgcca ttgagaagtt 20

<210> 7
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PRIMER

<400> 7
gaggcgattc ggctatgact g 21

<210> 8
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PRIMER

<400> 8
atcgggagcg gcgataccgt a 21

<210> 9
<211> 14
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:ADAPTER

<400> 9
aattgcaccg gtcg 14

<210> 10
<211> 10
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:ADAPTER

<400> 10
gatccgaccg 10

<210> 11
<211> 10
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:ADAPTER

<400> 11
tagccatggg 10

<210> 12
<211> 16
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:ADAPTER

<400> 12
tcgacccatg gctaat 16

<210> 13
<211> 35
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PRIMER

<400> 13
cccgtcgact ttccccgatc gttcaaacat ttggc 35

<210> 14
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PRIMER

<400> 14
cccggatcca aaaatggtga cagtcgagg 29

<210> 15
<211> 31
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PRIMER

<400> 15
ccggtcgacg caaatacatt catggcaaac g 31

<210> 16
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PRIMER

<400> 16
ggcggatcca aaaatgtctc ctccagtgtc 30

<210> 17
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PRIMER

<400> 17
cccgtcgacc taaactagac tccaatcact 30

<210> 18

<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PRIMER

<400> 18
cggggatcca gagggcctaa cttctgtata gac 33

<210> 19
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PRIMER

<400> 19
cccgtcgact cgcgaagata tagctaatcg 30

<210> 20
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:ADAPTER

<400> 20
aattgggcgc gccaagcttc cgaattctta attaag 36

<210> 21
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:ADAPTER

<400> 21
agctcttaat taagaattcg gaagcttggc gcgccc 36

<210> 22
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PRIMER

<400> 22
cccatcgatg cgtctagtaa catagatgac 30

<210> 23
<211> 91
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:MULTIPLE
CLONING SITE

<400> 23
ggcgcgccaa gcttgcatgc atcgatatgg tcgactctag aggatccccg ggtaccgagc 60
tcgaattcca gatctgcggc cgcttaatta a 91

INTERNATIONAL SEARCH REPORT

Int. tional Application No
PCT/EP 99/05027

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 C12N9/90 A01H5/08 A01H5/10 A61K35/78
A23L1/24 A23L1/39 A61K7/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 335 451 A (VERENIGING VOOR CHRISTELIJK WETENSCHAPPELIJK ONDERWIJS) 4 October 1989 (1989-10-04) column 12, line 10 - line 45 column 12, line 56 -column 14, line 32; figure 1 — -/—	1-3,14, 15,18, 20,21, 24,25

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 December 1999	13/12/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	De Kok, A

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inte ional Application No
PCT/EP 99/05027

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	VAN TUNEN A J ET AL: "REGULATION OF CHALCONE FLAVANONE ISOMERASE (CHI) GENE EXPRESSION IN PETUNIA HYBRIDA: THE USE OF ALTERNATIVE PROMOTERS IN COROLLA, ANTHERS AND POLLEN" PLANT MOLECULAR BIOLOGY., vol. 12, no. 5, 1 May 1989 (1989-05-01), pages 539-551, XP000381739 DORDRECHT NL	20
A	the whole document	1-3, 10-12
X	WO 94 03606 A (INTERNATIONAL FLOWER DEVELOPMENTS PTY. LTD.) 17 February 1994 (1994-02-17) page 3, line 7 -page 11, line 7	22
A	GROTEWOLD E ET AL: "ISOLATION AND CHARACTERIZATION OF A MAIZE GENE ENCODING CHALCONE FLAVONONE ISOMERASE" MOLECULAR AND GENERAL GENETICS., vol. 242, 1994, pages 1-8, XP002049933 BERLIN DE	1,2
A	MEHDY ET AL: "STRUCTURE OF AN ELICITOR-INDUCIBLE CHALCONE ISOMERASE GENE IN PHASEOLUS VULGARIS" EMBL SEQUENCE DATABASE, 5 September 1992 (1992-09-05), XP002032749 HEIDELBERG DE * Accession Nr.: Z15046 * abstract	1,2
A	WO 90 11682 A (DNA PLANT TECHNOLOGY CORPORATION) 18 October 1990 (1990-10-18) page 5, line 1 -page 14, line 35	1
P,X	WO 99 14351 A (DU PONT) 25 March 1999 (1999-03-25) page 2, line 7 -page 3, line 27 page 11, line 7 - line 18	1,2,14, 18,20-22

1

INTERNATIONAL SEARCH REPORT

information on patent family members

Inte ional Application No
PCT/EP 99/05027

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
EP 0335451	A	04-10-1989	NL	8800756 A	16-10-1989
			JP	2016985 A	19-01-1990
WO 9403606	A	17-02-1994	AU	667392 B	21-03-1996
			AU	4690193 A	03-03-1994
			CA	2140770 A	17-02-1994
			EP	0656951 A	14-06-1995
			JP	7509606 T	26-10-1995
			NZ	254673 A	28-05-1996
			US	5859329 A	12-01-1999
WO 9011682	A	18-10-1990	US	5034323 A	23-07-1991
			AT	123806 T	15-06-1995
			AU	640644 B	02-09-1993
			AU	5412390 A	05-11-1990
			DE	69020151 D	20-07-1995
			DE	69020151 T	28-09-1995
			DK	465572 T	07-08-1995
			EP	0465572 A	15-01-1992
			EP	0647715 A	12-04-1995
			ES	2075897 T	16-10-1995
			JP	4504800 T	27-08-1992
			WO	9012084 A	18-10-1990
			US	5231020 A	27-07-1993
			US	5283184 A	01-02-1994
WO 9914351	A	25-03-1999	AU	9493498 A	05-04-1999